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THE FOLLICULINIDÆ (INFUSORIA HETEROTRICHA) OF THE BRETON COAST

E. FAURÉ-FREMIET

(From the Collège de France, Paris, France)

The Infusoria of the family Folliculinidæ, which nearly all inhabit salt water, are widely distributed along the coasts. Their identification is made with difficulty by means of the tube or lorica, flask or bottle-shaped, which is the principal character employed in this case. On the Breton coast, in the bay of Concarneau, I found 15 different species. The observation of these species, in a living state or after fixation, allows one to define with precision the characteristics of species already known (2 only seemed new). It seems necessary, in spite of the excellent works of C. Dons and Kahl, to consider a revision of the group as a whole. This will be the subject of a more important study; the present paper's aim being only to give a brief description of the species found at Concarneau. As a matter of fact, I have been obliged to give particular consideration to the shape of the macronucleus. This may vary: it may be moniliform and beaded, or single, ovate or spheroidal. It seems necessary to divide the genus *Folliculina sensu stricto,* and I propose the new genus *Folliculinopsis* for the species endowed with a beaded macronucleus, the primitive generic denomination of *Folliculina* being kept for species with a single ovate macronucleus. This allows one to complete the subdivisions of the genus *Folliculina* (primitive sense) for which Dons has created several new genera.

The following descriptions are based only on the settled individuals; the swimming form has been observed for nearly all these species, but its specific characters are not sufficiently well marked to be very useful in their identification.[1]

[1] For bibliography, refer to:

Kahl, A., 1932. Wimpertiere oder Ciliata, in *Die Tierwelt Deutschlands,* G. Fischer, Jena.

Kahl, A., 1933. Ciliata libera et ectocommensalia, in *Die Tierwelt der Nord- und Ostsee,* Leipzig.

Fauré-Fremiet, E., 1936. La Famille des Folliculinidae, in *"Mélanges Paul Pelseneer"* (in press).

Folliculinopsis producta Wright (Fig. 1)

This species is very common and is usually fixed on various objects (algæ, shells, etc.) in the intercotidal zone. It is easily cultivated in aquaria. The infusorian, fully expanded, is more than 1 mm. long. The tube is attached laterally for a distance of nearly 200 μ, then it bends almost at a right angle and takes the shape of a spirally marked neck (500 to 600 μ) slightly everted at its margin.

The body of the fully expanded infusorian is elongate, slender, growing very thin towards its posterior end. The two peristomal lobes are long and narrow, nearly always standing straight in the prolongation of the body. The macronucleus is made up of about 20 beads. The color of the infusorian is dark green-blue.

Folliculinopsis annulata sp. nov. (Fig. 2)

Frequently found in colonies of Bryozoa, at depths of 15 to 20 meters. The tube-length attains or surpasses 800 μ; it is composed of a flat-lying lodge, adherent, which bends upwards into a long neck, often flexuous, with annular thickenings, of which the base always shows a kind of inner collar, short and in the shape of a truncate cone.

The infusorian has never been observed more than half expanded, with the peristomal lobes elongated in parallel lines within the lorica's collar.

Folliculinopsis lignicola sp. nov. (Fig. 3)

The shape of the infusorian is very much like that of *F. producta,* but fully extended its length does not exceed 350 μ. The macronucleus consists of a dozen beads; the infusorian has a pale blue color. This small species seems to be associated with *Limnoria lignorum,* for it builds its rudimentary shell in the pitted tracheids which are cut through by the hollowed galleries of these isopods in the pine wood of submerged wrecks.

Folliculinopsis gunneri C. Dons (Fig. 4)

The blue-green, fully expanded infusorian measures almost 400 μ; the shell, lying flat, is continued by a wide, slightly upturned neck, everted at its margin, often irregular; its wall is thick and brownish. This species resembles *Folliculina aculeata* in its general aspect, but differs from it by being smaller in size and having a moniliform macronucleus of 7 to 8 beads. It has been encountered in Concarneau, not on the integument of *Limnoria,* as was observed by Dons, but on the surface of galleries hollowed by this isopod.

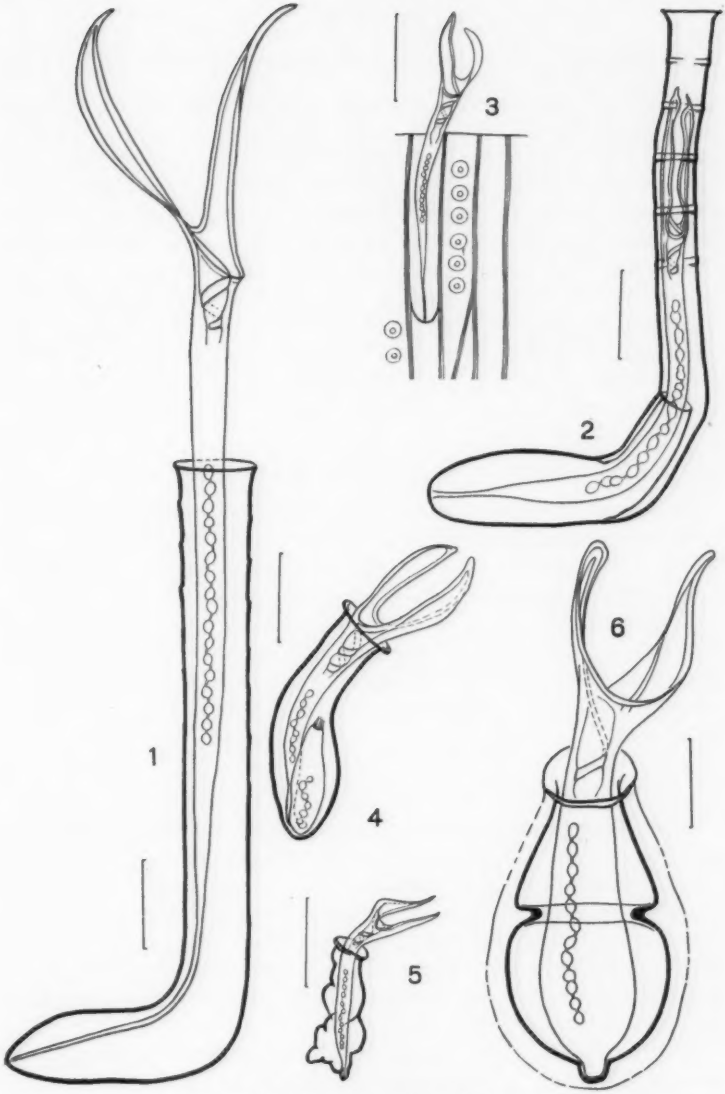

FIG. 1. *Folliculinopsis producta.*
FIG. 2. *Folliculinopsis annulata.*
FIG. 3. *Folliculinopsis lignicola.*
FIG. 4. *Folliculinopsis gunneri.*
FIG. 5. *Folliculinopsis limnoria.*
FIG. 6. *Pebrilla paguri.*
Scale indicated = 100 μ.

Folliculinopsis (*Mirofolliculina* Dons) *limnoriæ* Giard (Fig. 5)

This small species, always fixed on the last segments of *Limnoria lignorum*, is well characterized by the very irregular shape of its shell, which is 130–170 μ long. This is terminated by a very short collar, and spreads out to a large size with finger-like prolongations which may be extremely variable from one individual to another. Dons has established the genus *Mirofolliculina* for this single species; but the changing irregularity of the tube is a character of secondary importance. The expanded infusorian is 210–230 μ long; the beaded macronucleus (insufficiently observed by Giard) obliges one to rank this species within the genus *Folliculinopsis*, near to *F. gunneri*, from which it is distinguished first of all by a marked difference in size and by its specific association with *Limnoria lignorum*.

Folliculinopsis (*Pebrilla* Giard) *paguri* Giard (Fig. 6)

The genus *Pebrilla* Giard has been established for this single species of which the shell lies attached on the integument of *Eupagurus bernhardus*. This shell shows a very short and widely everted collar, a very pronounced transversal median wrinkle and a posterior stud.

The fully expanded infusorian approaches 600 μ in length, the shell 360–400 μ. I have frequently found this large and very characteristic species on the abdomen of *Eupagurus bernhardus* and of *Clibanarius misanthropus*. The macronucleus is always moniliform and shows a dozen beads.

Folliculina elegans Clap. and Lach. (Fig. 7)

This species, existing in great numbers on the Atlantic coast, attaches itself on various objects and is easily cultivated on submerged glass plates. I studied it at Concarneau and at Woods Hole. It certainly has often been described under the specific name of *ampulla;* but confusion is here impossible, as the macronucleus is single and oval, while the two folliculines described by Kahl under the names of *Ampulla* Kahl and *Möbiusi* Möbius [2] show a beaded macronucleus and are to be classified in the genus *Folliculinopsis*.

The tube consists of a horizontal lying lodge 240–270 μ in length and of a neck bending up at an angle of about 45°, slightly everted at its margin. The expanded infusorian is nearly 600 μ long, the posterior part enlarged, straightened into a long neck which precedes the peristome; both lobes run into a short sharp end.

[2] I did not observe these forms at Concarneau.

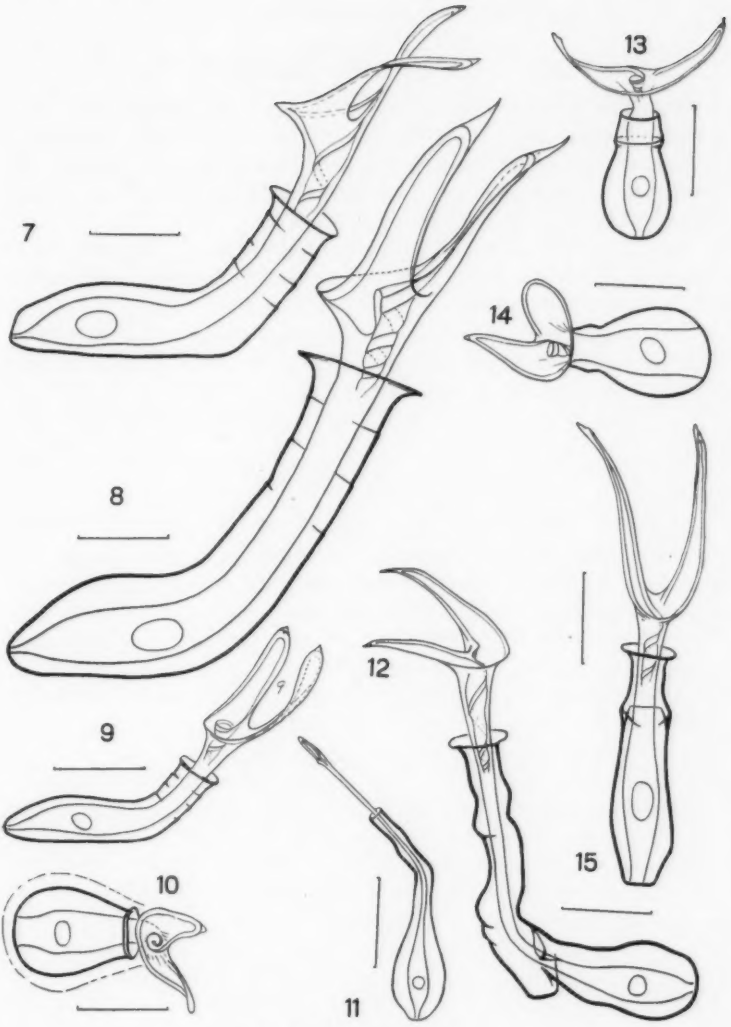

FIG. 7. *Folliculina elegans.*
FIG. 8. *Folliculina aculeata.*
FIG. 9. *Folliculina viridis.*
FIG. 10. *Folliculina simplex.*
FIG. 11. *Folliculina spirorbis.*
FIG. 12. *Parafolliculina mirabilis.*
FIG. 13. *Parafolliculina hirundo.*
FIG. 14. *Parafolliculina amphora.*
FIG. 15. *Parafolliculina violacea.*
Scale indicated = 100 μ.

Folliculina aculeata Clap. and Lach. (Fig. 8)

This species, very close to the above, is distinguished by its considerably greater size, by the development of its pharyngeal cavity and by the two acute points, protoplasmic, rigid, and very accentuated which terminate the peristomal lobes, answering completely to the description given by Claparède and Lachmann.

The infusorian measures, expanded, approximately 800 μ; the tube exceeds 550 μ; horizontal at first, it progressively bends upwards and is widely everted into a collar.

F. aculeata can be found at Concarneau at a depth of 20 meters on shells and on Bryozoa. It is probably this same species which has been described by Giard under the name of *F. abyssorum; F. similis* Dons and *F. gigantea* Dons may be synonyms, as well as *F. stylifera* Wright.

Folliculina viridis Wright (Fig. 9)

One can possibly identify with the species briefly described by Wright a small *Folliculina* whose form is very close to the two species just described, but which is very distinctly distinguished and in a constant way, by its smaller size and by its yellow-greenish or green color, very different from the blue-greenish or gray-bluish colors of all the other species.

The length of the infusorian reaches almost 400 μ, that of the lorica, 250 μ.

Folliculina simplex Dons (Fig. 10)

This tiny *Folliculina* is one of the species which have been at first associated under the name of *F. ampulla* O. F. Müller[3]; the denomination of *simplex,* which is Dons' creation, stretches out, it seems, over a group of species, distinct but very difficult to identify, because of lack of characteristic details. Various forms observed both at Concarneau and at Woods Hole seem to differ, in fact, in quite a constant manner, it may be in the shape of the constantly very short collar, or in the abundance of the blue-greenish pigment and its repartition. The only *Folliculina* known in fresh water, *F. boltoni* Kent (*Ascobius lentus* Henneguy) differs from *F. simplex* by its lodging only.

The expanded infusorian measures from 190 to 220 μ; the peristomal lobes are broad and short. The shell is patulous and measures 135–175 μ. *F. simplex* is easy to cultivate on submerged glass plates.

[3] I first admitted this denomination, in 1932, following the description of Clap. and Lach.

Folliculina spirorbis Dons (Fig. 11)

This very characteristic species, widely distributed, which attaches itself exclusively to the tubes of *Spirorbis*, is very difficult to observe, and therefore has been described by its shell only. The shape of this shell is a kind of ampulla of about 100 μ in length, prolonged by a long and very narrow tube (11 to 12 μ), which is erect and often flexuous. The infusorian is characterized by its simplified structure; fully expanded it shows an oval body going over into a slender and very long neck, terminated by a small peristome of which the left lobe only is developed.

Genus *Parafolliculina* Dons

Macronucleus ovoidal. Shell showing an annular wrinkle, as a separation between the single plain posterior lodge and the fore-chamber, placed at the base of the neck.

Parafolliculina mirabilis Dons (Fig. 12)

In *P. mirabilis* the tube is distinctly and constantly divided in three parts: a posterior ampulla, a widened entrance and a tubular and irregular neck, sometimes bent upwards, largely everted at its margin. The total length is over 400 μ.

The expanded infusorian measures almost 600 μ; in its general shape it looks very much like *Folliculina elegans*, but the neck, which is elongate between body and peristome, is very long and slender.

This large species, very characteristic, is found in Concarneau at a depth of 15 to 20 meters in colonies of *Membranipora* and in the company of *Folliculinopsis annulata*. It may be cultivated in aquaria on immersed glass plates.

Parafolliculina hirundo Kent (Fig. 13)

P. hirundo is very similar to *Folliculina simplex*, but its horizontal shell, with a length of about 130 μ, is a trifle straighter, with an annular wrinkle limiting an anterior entrance which is but one with the very short neck. The peristomal lobes are narrow and lie relatively far apart. This small species, feebly colored, has been observed occasionally on immersed glass plates.

Parafolliculina amphora Dons (Fig. 14)

This species is distinguished from the above by the shell's aspect only, the posterior end being broader and the peristomal lobes elongated. I

occasionally observed it, as well as *P. hirundo*, on immersed glass plates in aquaria, at Concarneau and at Woods Hole, Mass. The observations made on these two neighbouring forms are not sufficient to enable one to decide whether or not they should be separated into two distinct species.

Parafolliculina violacea Giard (Fig. 15)

This particularly typical and widely distributed species has been described by Laakmann (1903) and by Dons (1912) under the name of *Folliculina telesto*. The tube stands straight, about 250 μ long; the widened fore-chamber is continued by a rather short neck, everted at its margin; the entrance shows a kind of inner collar at its base.

The expanded infusorian is 500 μ long; the peristomal lobes, long and slender, stand straight and parallel to the axis of the body; the color is reddish-violet.

AN EXPERIMENTAL STUDY OF POLYSPERMY

JEAN M. CLARK

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Although the mechanism which insures that only one sperm shall fertilize an egg is most closely concerned with the fertilization reaction as a whole, it has been very little investigated. This is largely due to the fact that no visible alteration in the surface protoplasm can be associated with the change to a fertilized condition, and the failure of the mechanism, as it appears in polyspermy—the entrance of more than one sperm into an unfertilized egg—is not easily detectable in the living condition until the time of the first cleavage. With the exception of the pioneer work on polyspermy, performed on the eggs of *Strongylocentrotus lividus* by O. and R. Hertwig in 1887, there has been little or no direct study of the phenomenon either from an observational or an experimental point of view. Numerous embryologists have been incidentally concerned with polyspermy as it appeared in the course of their work, and most of the information which has been added to the Hertwigs' contribution has come through such indirect channels. The summarized results of some of these studies are included later with the discussion of the effective agents for inducing polyspermy.

Speculation as to the nature of the mechanism which prevents polyspermy has not been wanting, but only one proposition has been supported by a majority of the authorities in the field. This explanation, embodying the conclusions reached by O. and R. Hertwig (1887), E. B. Wilson (1903), Delage (1901), Godlewski (1926), F. R. Lillie (1919) and Just (1929), while it is actually little more than a restatement of fact, includes the major points which may be deduced from the accumulated evidence. Briefly, it states that a change in the egg protoplasm, probably in the cortical region, initiated by the fertilizing sperm, renders the egg inaccessible to further sperm. It is generally agreed, moreover, that polyspermy is caused by a retardation in the rate at which this change is propagated around the egg from the entrance point of the sperm.

Until the present, this latter proposition has stood unchallenged by experimental procedure, although it is actually only a deduction from scattered qualitative observations. While it is admittedly the

simplest and most easily visualized mechanism which can be postulated to explain the observed effects, such reasoning is insufficient foundation for the confidence and lack of critical examination which it has enjoyed. Even such preliminary experimentation as is reported in this paper leads to results which are exceedingly difficult to reconcile with the idea of the retardation of a propagated change in the egg cortex as the cause of polyspermy. It is entirely possible that the true explanation may have an entirely different basis, and this possibility should at least be kept under consideration.

In view of the fact that monospermy is one of the few general characteristics of fertilization which are capable of manipulation without immediately fatal consequences, it is rather remarkable that so little attention has been given to its study as a method of investigating the fertilization reaction. At the suggestion and under the direction of Dr. L. V. Heilbrunn, the following experiments were undertaken to repeat with the eggs of *Arbacia punctulata* the work done by the Hertwigs (1887) on *Strongylocentrotus*. Their original list of agents was extended to include a wider range of chemical substances, and a few studies were made on other species for comparison.

General Method

In general, one procedure was used in all the succeeding experiments, with slight modifications in certain cases. The eggs and sperm were obtained from the animals according to the directions given by E. E. Just (1929). The oral surface of the sea urchin was cut, the lantern removed, and the male animals inverted over dry Syracuse watch glasses, while females were allowed to shed into Stender dishes containing sea water. After being washed several times in sea water, some of the eggs from each female were tested by insemination. Such lots as gave 100 per cent entirely normal fertilization reaction were used for experimentation. In some cases several lots were mixed (usually eggs from 2–5 females) in order to reduce the variation in the results arising from individual differences characteristic of the parent animals.

The number of sperm used for insemination in each experiment was controlled so far as possible by making a standard suspension consisting of one drop of "dry sperm" in 25 cc. of sea water. From 2–5 drops of this suspension were used to fertilize each lot of eggs in the experiments. Although this method gives only an approximately uniform insemination, experiments showed that even a much wider range of variation had in itself no effect in changing the number of sperm which entered each egg. Except in experiments involving

over-insemination, only enough sperm suspension was used to produce a moderate insemination—approximately 1/20 of the sperm concentration which was necessary to secure 10 per cent polyspermy in otherwise untreated *Arbacia* eggs.

In the case of each reagent, tests were made with dilute sperm suspensions to make sure that the sperm retained their complete capacity for fertilization after exposures of at least 5 minutes. It was assumed that within this time all fertilizable eggs in a well-mixed sperm and egg suspension would be fertilized.

The general procedure in all experiments was as follows: eggs and sperm were shed, and the eggs tested for 100 per cent normality. Several drops of concentrated egg suspension were introduced into the test solutions and left for varying periods. The eggs were inseminated in the test solution, or they were removed to sea water containing sperm, or to sea water to which sperm were added after a short recovery period. Controls were inseminated in sea water to check the incidence of polyspermy resulting from injury incurred during the handling of the eggs. In almost no case was this higher than 1–2 per cent, and a majority of the cases showed none at all.

The eggs were allowed to develop to the first cleavage, and the percentages of development (i.e., partial or complete fertilization—raised fertilization membrane, characteristic change in pigmentation, or visible nuclear activity) and of polyspermy (cleavage into three or more blastomeres or appearance of three or more daughter nuclei in an uncleaved egg after an hour) were recorded. The percentage of development represents the ratio of activated eggs to all the eggs in the suspension. The figures for polyspermy, on the other hand, give the percentages of *activated eggs* which were polyspermic.

In certain cases in which the test solution was strongly toxic it was removed after 10–15 minutes and fresh sea water was added, to allow the development of the eggs to proceed. This was done only when sufficient time had elapsed after insemination so that all the sperm in the suspension had been deprived of fertilizing power.

In these experiments, although the temperature varied during the season from 18.0° to 28.0° C., most of the work was done at about 23.0° C. In any one experiment all the solutions were allowed to come to the same (room) temperature before the eggs were introduced into them. Undoubtedly some of the variation observed in the results may be traced to temperature effects; in order to separate out such variation from that due to individual differences, it will be necessary to perform a series of experiments using eggs from the same females at extremes of the temperature range. In the meantime no consistent effect can be detected in the data at hand.

All alkaloid solutions were made up in fresh sea water, checked for pH with Clark and Lubs indicators, and kept in a refrigerator between experiments to prevent decomposition of organic constituents. Other conditions, such as amount of solutions and number of eggs, were kept relatively constant by the use of Stender dishes and pipettes of uniform size, and results were checked with controls whenever this was possible.

Specific Differences in Susceptibility to Polyspermy

It is generally conceded throughout the literature that insemination with an excess of sperm will cause polyspermic fertilization in the eggs of many marine animals, even when these eggs are in good condition. Just (1929), on the other hand, directly contradicts this. In deciding the issue, there is always the difficulty of determining the point at which the concentration of sperm begins to inflict injury upon the egg cells by accumulation of CO_2 and probably other substances. In *Arbacia* such injury is very apparent with extremely heavy insemination, causing marked irregularities in development. The polyspermy which appears with moderately heavy sperm concentrations may well be the first evidence of the injury. On the other hand, great differences in the susceptibility of eggs to polyspermic fertilization can be demonstrated between different species. *Cumingia* eggs, for example, are strongly affected by sperm concentrations which would give no polyspermy in *Arbacia*, and which, on microscopic examination, show no crowding of sperm about the egg peripheries.

A technique for exact comparative study of different species would require the development of a special method for counting with some accuracy the number of sperm in a given suspension. There are, moreover, other factors, such as size of eggs, size and shape of sperm, and the precise mechanism for sperm entrance into the eggs, which contribute to the final result. With these qualifications in mind, a simple method was used to give approximate values for each of the four species studied. For *Arbacia punctulata* and *Asterias forbesii*, sperm suspensions could be made roughly comparable by using the same dilutions of dry sperm. Preliminary experiments indicated the sperm concentration which uniformly gave 100 per cent fertilization with the lowest percentage of polyspermy to be 0.06 cc. of the "standard suspension" made by mixing one drop of dry sperm with 25 cc. of sea water. With lighter insemination not all the eggs were fertilized; when the amount of sperm suspension was considerably increased (ca. 10 times), polyspermy became slightly more frequent. The sperm of *Cumingia tellinoides* and *Cerebratulus lacteus* cannot be obtained dry; the procedure used in these cases was to determine the

lowest concentration which consistently produced 100 per cent development when the eggs were inseminated with 0.06 cc. of it. This then formed the unit suspension of which increased amounts were used to produce polyspermy.

Eggs and sperm of *Arbacia* were obtained in the usual manner. *Asterias* eggs were procured by removing one-half of a ripe ovary to a finger-bowl filled with sea water. After 5 minutes the ovary was removed; the eggs which had been extruded were allowed to remain in the sea water for from 30 to 55 minutes to permit the breakdown of the germinal vesicle. Sperm were shed into a dry Syracuse watch glass from the excised half-testis, and the test suspensions made from this concentrated stock. *Cumingia* eggs and sperm were shed into Stender dishes of sea water into which the animals were placed after being kept dry for a short time. *Cerebratulus* gametes were teased out of sections cut from the sexually mature worms. In order to permit a rough comparison of the results, the sperm concentration obtained by adding 0.06 cc. of the "standard suspension" to an approximately equal number of eggs in 30 cc. of sea water is considered as unity in Fig. 1. The eggs were inseminated with multiples of this amount to 100 times the unit suspension or until the incidence of polyspermy approached 100 per cent.

The curves for the four species are plotted in Fig. 1. It is at once apparent that there are wide differences in the order of susceptibility to polyspermy among these species. When the unit concentration of *Cerebratulus* sperm is increased 20 times, these eggs are nearly all polyspermic. Because of the difficulty of obtaining eggs and sperm of this animal, the experiment was performed only once, and the result must be considered as simply indicative. *Cumingia* eggs are 100 per cent polyspermic with 50 times the original sperm concentration, while a sperm suspension 75 times more concentrated than the minimum gave only 41 per cent polyspermy in *Asterias* and 14 per cent in *Arbacia* eggs. No correlation can be found between the size of the egg and susceptibility to polyspermy, the size series being *Cerebratulus*, *Asterias*, *Arbacia*, *Cumingia*. It is most probable that the explanation of this disparity must be sought, not in morphological or simple physical and chemical variations, but in the less easily defined physiological differences between various types of eggs, such as their resistance to deterioration after exposure to sea water, and the influence which is exerted on the cytoplasm by the stage of the maturation process in which the eggs are fertilized.

Age

Preliminary studies of the effect of aging on *Arbacia* and *Asterias* eggs were undertaken to furnish data for comparison between the two

forms, and also to attempt to find a basis for explanation of the fact that Smith and Clowes (1924), in their pH work, obtained 100 per cent polyspermy in *Asterias* at the pH of sea water. In these experiments no attempt was made to control temperature or to check bacterial action, since the aim was not to differentiate between the rôles of various factors in the aging process, but to discover their combined effect on the fertilization reaction.

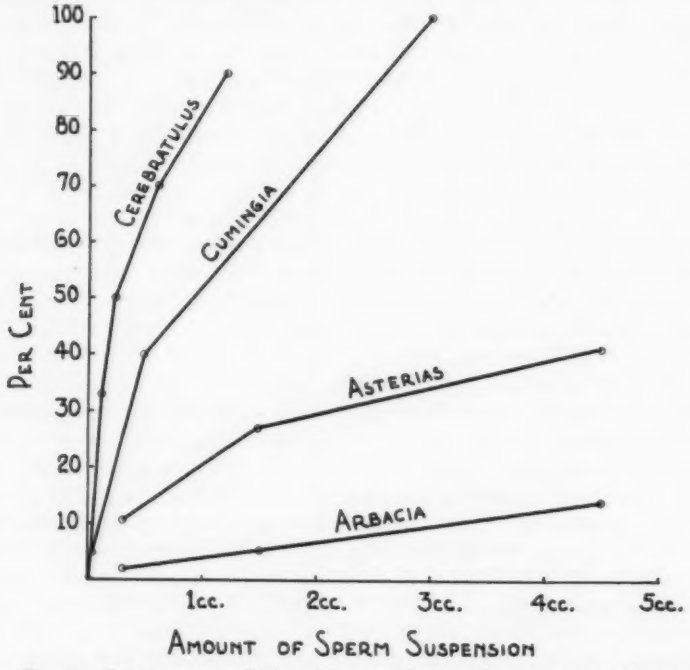

FIG. 1. Relative susceptibility of four species to polyspermy caused by over-insemination.

Eggs were kept in uncovered finger-bowls filled with sea water, in dilute suspension to avoid undue crowding, at room temperature. They were removed at intervals to Stender dishes and inseminated with freshly secured sperm in the usual concentration.

Arbacia eggs uniformly showed no diminution in their resistance to polyspermy with moderate insemination until they had been standing at least 16 hours in sea water. The percentage of polyspermy then increased regularly until at 23 hours it occurred in all eggs

which were still capable of fertilization. The eggs of *Asterias* presented a very different picture, which is represented in graphic form in Fig. 2. These curves, which indicate the increased polyspermy with increased time after shedding of the eggs into sea water, include the results of 5 experiments in which eggs were inseminated with the usual light sperm suspension, and simultaneously with one 80 times more concentrated. When eggs are inseminated lightly 45 minutes

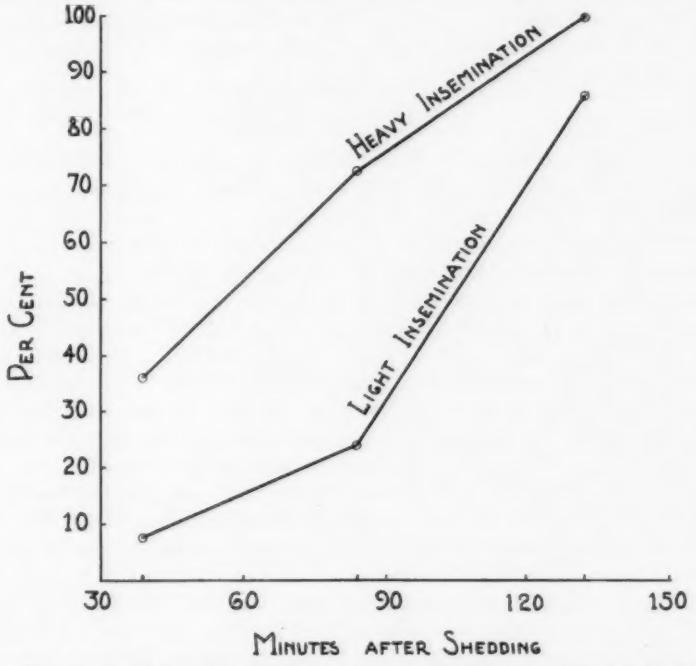

FIG. 2. Increasing susceptibility to polyspermy with increased time of standing in sea water, in the eggs of *Asterias forbesii*.

after exposure to sea water, the polyspermy is somewhat less than 10 per cent; after exposures of 85 minutes to sea water, the polyspermy increases to 24 per cent. Between 2 and 2½ hours after shedding, 86 per cent of the eggs are polyspermic, even though very low concentrations of sperm are used. With heavy insemination, there is 36 per cent polyspermy at about 45 minutes, 73 per cent after 85 minutes, and 100 per cent between 2 and 2½ hours. Microscopic examination of heavily inseminated eggs immediately after insemina-

tion showed the jelly surrounding the eggs to be completely filled with active sperm. This degree of insemination must be comparable to that designated by Smith and Clowes as "maximum" since it approaches the physiological limit—further increase in the number of sperm causes injury apart from polyspermy.

The rapidity with which *Asterias* eggs lose their capacity to resist polyspermy after exposure to sea water and completion of the maturation process suggests that the high percentages of polyspermy found by Smith and Clowes are due to the use of eggs which had stood in sea water too long (i.e., 1½–2 hours) after shedding to be in optimum condition for fertilization.

Hertwig Reagents

In repeating with *Arbacia punctulata* the Hertwig (1887) experiments performed on the eggs of *Strongylocentrotus lividus* (*Paracentrotus lividus*), no attempt was made to determine the optimum concentration or length of exposure for the production of polyspermy with the particular substance used, chiefly because the present state of the knowledge both of the mode of action of the complex chemicals involved and of the cell mechanism affected by them is too fragmentary to make profitable use of detailed quantitative data. In each case the concentrations selected in the original work were used, although the time of exposure was varied to some extent. In order to overcome the fluctuations introduced by individual variation, the data presented include results of several experiments performed on different lots of eggs. Except in cases in which the percentages were close to 0 or 100, the final average is made from individual experiments, the results of which may vary widely. Indeed, the average for one lot of eggs may be ± 30 per cent from the final average.

The substances which were selected, somewhat fortuitously, by the Hertwigs for their experimental studies of fertilization fall rather clearly into three categories so far as their action on *Arbacia* egg cells is concerned. Chloroform, which is in a class alone, will be discussed below. Of the others, chloral hydrate, cocaine hydrochloride and nicotine form one group, and morphine sulphate, strychnine sulphate and quinine sulphate, another.

The action of the first group is characterized in general by its lack of effect in entirely blocking fertilization, its production of a high percentage of polyspermy, and its immediate and nearly complete reversibility on return of the eggs to sea water for insemination. The substances in the second group tend to reduce the percentage of development; they induce less polyspermy, and the polyspermy-inducing

action is not so rapidly reversed on return to sea water, although the block to fertilization is removed. (See Table I.) Chloral hydrate, cocaine hydrochloride and nicotine, in concentrations which will give 100 per cent polyspermy, permit development to proceed at least past the first few cleavages in the test solutions, while morphine, strychnine and quinine sulphates are rapidly (within 30 minutes after insemination) fatal to eggs left to develop in the solutions, turning the protoplasm dark and apparently coagulating it. These facts seem to indicate a more or less specific attack, on the part of the

TABLE I

Comparative resumé of results obtained by treating unfertilized *Arbacia* eggs with reagents used by O. and R. Hertwig (1887).

Reagent	Inseminated in solution		Inseminated in sea water	
	Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Chloral hydrate, 0.2%	98	92	100	5
Cocaine HCl, 0.025%	95	80	100	19
Nicotine, 1 drop—200 cc.	94	73	100	9
Morphine SO_4, 0.6%	53	52	100	31
Strychnine SO_4, 0.1%	100	56	100	50
Quinine SO_4, 0.05%	76	31	100	76

substances in the first group, on the mechanism of monospermy, while the second group of substances injures the whole cell, and polyspermy is only one of the manifestations of its poisoned condition.

Although these common characteristics are apparent, the action of each of the reagents is sufficiently different from the others to warrant a brief individual description.

Chloral Hydrate

This substance, in 0.2 per cent solution, gives a higher percentage of polyspermy than any of the alkaloids, with apparently less injury to the cells, after 5 minutes exposure or less (see Table II). Its action is initiated very rapidly, as is shown by the fact that eggs introduced into a chloral hydrate solution containing sperm were 88 per cent polyspermic. It is very interesting that almost the maximum effect is obtained during the exceedingly brief interval which intervenes between contact of egg cell and solution and arrival of sperm at the egg surface. It is possible, in these experiments, that the sperm-excluding mechanism was secondarily broken down as a result of exposure to the solution, permitting the entrance of more sperm into

TABLE II

Results obtained from treatment with 0.2 per cent chloral hydrate.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	7	99	88		
1	7	98	97	100	3
5	7	96	97	100	7

normally fertilized eggs—however, there seems to be at present no method of checking this point. In preliminary experiments with nicotine, attempts to secure the entrance of extra sperm into normally fertilized eggs by exposing them to solutions of the drug a few minutes after fertilization gave negative results, even when the fertilization membranes were shaken off the eggs.

With a greater concentration of chloral hydrate—0.5 per cent—the effect on the cell is less specifically the production of polyspermy and more a general toxicity—i.e., the percentages of both development and polyspermy are reduced, and the immediate reversibility in fresh sea water is lost (see Table III).

TABLE III

Results obtained on treatment with 0.5 per cent chloral hydrate.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	6	35	61		
1	6	73	97	100	6
5	6	90	100	100	45
10	6			100	70

Cocaine Hydrochloride

Cocaine HCl in 0.025 per cent solution is similar to 0.2 per cent chloral hydrate in its action, giving high percentages of development and polyspermy. Its effect is found to be almost completely reversed when treated eggs are inseminated immediately after their return to sea water (see Table IV).

TABLE IV

Treatment with 0.025 per cent cocaine hydrochloride.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	18	90	51		
2	18	100	73	100	10
5	18	99	73	100	4
10	18	100	82	100	11

Nicotine

The nicotine solution was made by shaking one drop of nicotine with 200 cc. of sea water. In this concentration nicotine is almost identical with 0.025 per cent cocaine hydrochloride in effectiveness and reversibility (see Table V).

TABLE V

Results obtained on treatment with nicotine—1 drop of extract in 200 cc. sea water.

Exposure	Number of animals	Inseminated in solution		Inseminated in sea water	
		Percentage of all eggs activated	Percentage of activated eggs polyspermic	Percentage of all eggs activated	Percentage of activated eggs polyspermic
minutes					
0	7	80	56		
1	12	100	66	100	3
5	7	99	97	100	5
10	9	93	87	100	15

Qualitative experiments show that nicotine is effective over a very wide range of concentrations, from 1 drop in a liter of sea water to 50 drops in a liter. The close correlation between severity of treatment and the resulting degree of polyspermy is beautifully shown over this concentration range, although no quantitative measure can be given because of the impossibility of establishing exact grades of polyspermy. However, the progression is very apparent, from scattered cases of two-sperm fertilizations with the lowest concentrations of nicotine, through a point at which there is a general occurrence of di- and tri-spermy. Then, with stronger concentrations, fertilization uniformly involves 4 or 5 sperm, and finally, with very strong concentrations, there is entrance of many (ca. 20 or more) sperm into all eggs, with

TABLE VI

Eggs exposed to 0.6 per cent morphine sulphate for 15 minutes.

Inseminated in	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Solution	7	53	52
Sea water immediately after transfer	7	100	31
Sea water after 1 minute	7	100	1

subsequent cleavage into many blastomeres after the first hour, or entire suppression of cleavage, with the cells filled with sperm nuclei.

Low concentrations of nicotine solution (e.g., 3 drops in a liter of sea water) also permit clear demonstration of the reciprocal rôles of time and concentration. When eggs are exposed to a given solution for increasing periods, the same gradation of effect is obtained, although the extent of the range is usually restricted, i.e., with low concentrations even long exposure will not give the maximum effect, and the shortest possible exposure to a high concentration will produce more than the minimum effect.

Morphine Sulphate

In the second group of substances, morphine sulphate is most like the members of the preceding group in that there is some immediate recovery from its effects upon return of the eggs to sea water, and complete recovery takes place within one minute (Table VI). The percentage of development is, however, reduced to one-half when the eggs are inseminated in the solution, and polyspermic fertilization occurs at best in only about fifty per cent of these fertilized eggs. In order to obtain development as far as the first cleavage, it was necessary to remove the test solution and substitute sea water.

Strychnine Sulphate

The action of strychnine is less generally toxic than that of morphine, permitting 100 per cent fertilization (see Table VII), but its

TABLE VII

Eggs exposed to 0.1 per cent strychnine sulphate for 5 minutes.

Inseminated in	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Solution	13	100	56
Sea water immediately after transfer	13	100	50
Sea water after 1 minute	13	100	17

capacity for the production of polyspermy is less rapidly reversible in fresh sea water, showing almost no diminution on insemination in sea water immediately after transfer from the strychnine solution. There is only partial recovery of normal fertilizability after 1 minute.

Quinine Sulphate

The tendency toward irreversibility is carried to a remarkable extreme in the action of quinine sulphate, which shows a more than 100 per cent increase in the incidence of polyspermy when the eggs are transferred to sea water containing sperm, after 10 minutes or less exposure to a 0.05 per cent quinine sulphate solution (see Table VIII). Even after 1 minute of recovery in sea water, the percentage of polyspermy is higher than that obtained by insemination in the solution. This result is wholly unexpected and at present inexplicable.

TABLE VIII

Eggs immersed in 0.05 per cent quinine sulphate solution for 10 minutes.

Inseminated in	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Solution	12	76	31
Sea water immediately after transfer	16	100	76
Sea water after 1 minute	9	100	42

It is impossible to draw comparisons between these results and those of the Hertwigs because differences between the backgrounds of knowledge, the points of view and the methods employed in the two studies place the data in different categories. The most that can be said is that the agents studied affect the eggs of *Paracentrotus lividus* and of *Arbacia punctulata* in the same direction, and to an approximately similar extent.

FAT SOLVENTS

During the course of two summers, experiments were performed to test the action of fat solvents—ethyl alcohol, ether, chloroform and ethyl urethane—in causing polyspermy. It was expected that these substances would have well-marked effects because of their anæsthetic properties. Solutions were made up in every case immediately before their use, and kept covered while the eggs were being exposed to their action before insemination. The optimum concentrations for the production of polyspermy were: ether, 2.0 per cent; ethyl alcohol, 3.0–5.0 per cent; chloroform, 0.2 per cent; ethyl urethane, 2.0 per cent.

The results of these experiments were so varied that many more

data and more exact control of experimental conditions will be necessary before significant values can be obtained. From the results at hand, however, several facts are apparent. In the first place, all the substances tested are effective agents for the production of polyspermy, and, so far as could be determined, they possess roughly the same order of effectiveness. Experiments which involved insemination in sea water after exposure to the solution showed that the action of all the substances is reversed immediately upon return to sea water unless the treatment has been prolonged or severe.

Probably the greatest part of the observed variation can be ascribed to the temperature effect which is easily detectable in the data, although no experiments were planned to test this point. The fact that one concentration of ether or ethyl alcohol, over the temperature range from 22.0° to 27.0° C., caused successively no observable effect, 100 per cent polyspermy and 100 per cent cytolysis of the cell protoplasm demonstrates clearly that the treatment resulting specifically in the production of polyspermy possesses a very narrow range of effectiveness which lies only slightly below the lethal dose.

Common Cations

The extensive work which has been done by R. S. Lillie (1910), J. Loeb (1915), and others on the effects of the sodium, potassium, magnesium and calcium ions on egg cells and on the fertilization reaction suggested an attempt to discover the possible rôle of these cations in the production or prevention of polyspermy. Because they are constituents of the medium in which fertilization normally takes place, and because their properties are relatively well defined and their action on single cells has been extensively studied, they are more useful agents for experimentation on egg cells than the more complex and less familiar alkaloids. Several factors are involved, however, and these complicate the situation.

The greatest difficulty lies in the fact that the eggs cannot be fertilized in pure isotonic solutions of the chlorides of these ions because such solutions are highly toxic to both eggs and sperm. Moreover, it is impossible, without using a centrifuge to concentrate the eggs, to carry them from sea water to a pure chloride solution without including traces of the other salts of sea water. Centrifugal force was not used because of the danger of mechanical injury to the eggs.

With the possibility of inseminating in pure solutions ruled out, two alternative methods were used: first, exposure of eggs to chloride solutions for a relatively long time (20 minutes) and insemination immediately upon return to sea water; and second, the addition of an

excess of the isotonic salt solution to sea water and insemination in this mixture. Since the most drastic effect of isotonic solutions of sodium, potassium and magnesium on sperm motility depends on the lack of calcium, and since calcium is effective in extremely small quantities, the sperm retain their motility and fertilizing power in such solutions containing a great excess of one ion, which must then be considered to be exerting the predominant effect in influencing the course of the fertilization reaction.

Experiments with Isotonic Chloride Solutions

Arbacia eggs were shed into sea water and washed in the usual fashion; they were later transferred through three dishes of isotonic salt solution within twenty minutes, and inseminated immediately upon return to sea water. Although the shedding of the eggs directly into the salt solutions would have eliminated the necessity for repeated washings and insured entire absence of the other constituent salts of

TABLE IX

Unfertilized eggs washed for 20 minutes in three changes of isotonic solution, inseminated in sea water.

Cation	Number of experiments	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
Na	13	34	100	48
K	21	64	96	35
Ca	9	19	97	19
Mg	14	42	100	13

sea water, this procedure was not adopted because previous experimentation had shown that the percentage of development was much reduced in eggs which were not washed in sea water immediately after shedding and before other treatment was begun. In these experiments the eggs were concentrated as much as possible before transference from one solution to the next; however, it is certain that traces of all the common constituents of sea water were carried through the washings. The use of the hydroxides of sodium and potassium to raise the pH of calcium and magnesium solutions to that of sea water also introduced a slight error. The solutions, then, cannot be considered as quantitatively pure, but the marked differences found to exist between the actions of such solutions of the four common cations indicate that characteristic effects are being produced. Isotonic solutions were made with Merck's c.p. chemicals, NaCl and KCl in 0.53M solution and $CaCl_2$ and $MgCl_2$ in 0.32M solution. (The latter solutions were slightly hypertonic.) The pH was adjusted with 0.1N NaOH and KOH.

The results of these experiments are summarized in Table IX. It must be emphasized again that these figures are not indicative of the absolute magnitude of the polyspermy-producing action exerted by these reagents, since fertilization did not take place in the test solutions. They probably represent the amount of such effect which was not immediately reversed upon return to sea water, since, with the single exception of quinine sulphate, all the reagents tested gave a higher percentage of polyspermy when insemination took place in the solution than when the treated eggs were changed to fresh sea water containing sperm.

Experiments with Salt-Sea Water Mixtures

Mixtures of sea water and isotonic salt solutions were made in varying proportions, and eggs inseminated in these test solutions after an exposure of 10 minutes. A condensed comparison of the results obtained by this procedure with sodium, potassium, magnesium and calcium chlorides is presented in Table X. It is strikingly apparent,

TABLE X

Effect of 10-minute exposure to salt-sea water mixtures. Percentages represent salt solution/sea water ratio. *D* = percentage of all eggs activated; *P* = percentage of all activated eggs polyspermic.

Salt	No.	17%		33%		50%		67%		80%		93%	
		D	P	D	P	D	P	D	P	D	P	D	P
NaCl.....	12			100	2	100	0	94	0	*58*	0	10	3
KCl.....	12	*50*	0	31	0	0	—						
$CaCl_2$.....	7	94	5	62	1	*40*	2	8	6	0	—		
$MgCl_2$.....	60	100	5	98	9	93	17	98	37	87	71	*44*	32

from the distribution of the italicized values in the table indicating the approximate points at which the percentages of development were reduced by one-half, that the four cations have very unequally toxic effects on the fertilization reaction. This toxicity is not, however, clearly correlated either directly or indirectly with the capacity of the salts to induce polyspermy. It is perhaps significant that the least toxic, magnesium, produces the most polyspermy; on the other hand, sodium, which is only slightly more injurious than magnesium, is entirely ineffective so far as polyspermy is concerned. It is interesting, also, to note that this procedure gives results which are almost the opposite of those obtained by exposing eggs to solutions of single chlorides.

The data accumulated for magnesium in the course of a large number of experiments show that the concentration of magnesium (4 parts isotonic $MgCl_2$–1 part sea water) which causes the highest percentage of polyspermy is precisely the amount at which the fertilizability of the eggs begins to drop off sharply (see Fig. 3). This relation-

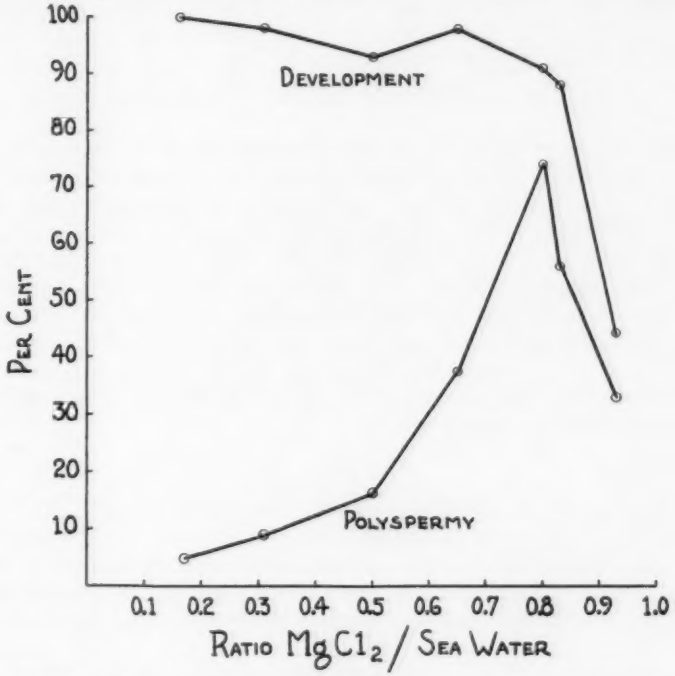

FIG. 3. Effect of various proportions of isotonic $MgCl_2$ and sea water on development and polyspermy in *Arbacia punctulata*.

ship points to the conclusion that the magnesium-sea water solution affects the sperm-excluding mechanism just before it fatally injures the cell as a whole.

In an attempt to discover why the proportion of one part sea water to four parts $MgCl_2$ was more effective than any other combination, a series of experiments was performed in which one part of isotonic NaCl, KCl and $CaCl_2$ respectively was added to four parts of $MgCl_2$. The results are shown in Table XI. The fact that the mixture of $MgCl_2$ and NaCl has an effect so similar to that of $MgCl_2$ and sea water is not surprising in view of the predominant amount of sodium in sea

TABLE XI

Unfertilized eggs exposed for 10 minutes to isotonic salt mixtures.

24 cc. $MgCl_2$ + 6 cc. of	Number of animals	Percentage of all eggs activated	Percentage of activated eggs polyspermic
NaCl	20	91	71
KCl	20	25	11
$CaCl_2$	20	22	50
Sea water	60	91	72

water. With the small traces of calcium introduced into the solution as part of the sea water in which the eggs were suspended, the final solution contains the cations necessary for fertilization. From the work of Fukuda (1935) it appears that characteristic changes in the permeability of sea urchin eggs to water follow exposure to mixtures of sodium and magnesium. It is highly probable that other functions of the cell protoplasm besides its permeability to water are affected by such ionic interaction, and it is entirely possible that the conditions leading to polyspermy may depend on similar combination effects. This seems the more probable when the ineffectiveness of magnesium chloride alone is considered.

pH

Because Smith and Clowes (1924) have found changes in the pH of sea water to be an exceedingly effective method of causing polyspermic fertilization, a series of experiments was performed on the eggs of *Arbacia punctulata* to repeat this work with the same procedure as that used in the preceding studies, in order to obtain data which

TABLE XII

Effect of 10-minute exposure to sea water at lowered pH. Eggs inseminated in pH solutions.

pH	Number of experiments	Percentage of all eggs activated	Percentage of activated eggs polyspermic
5.8	5	4	20
6.4	9	49	46
7.0	24	91	78
7.6	9	97	50
8.2	9	99	7
Sea water	24	99	0–2

would be strictly comparable with the values obtained for the other agents. This work differed from Smith and Clowes' in that counts were made at the time of the first cleavage instead of 2–4 hours after insemination, and treated eggs were inseminated with a light suspension of sperm (see above) instead of the "excess sperm" used by Smith and Clowes. In other respects their technique was followed

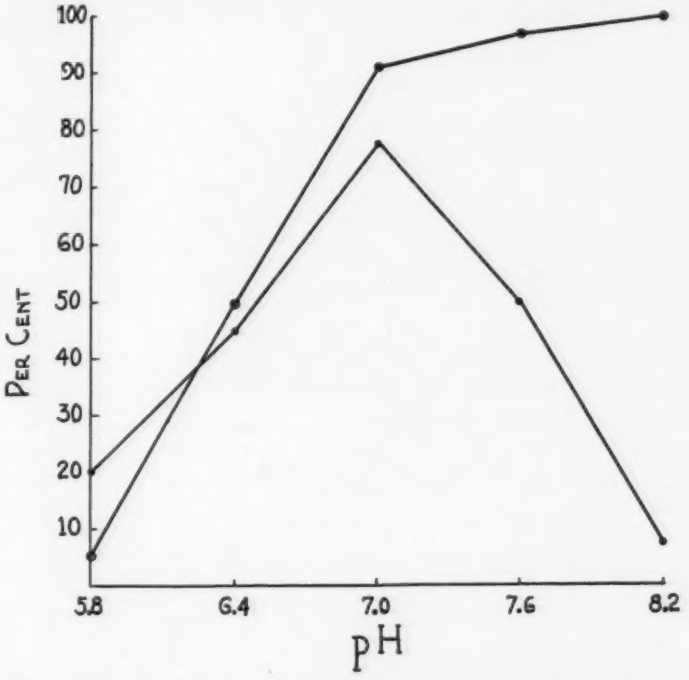

FIG. 4. Effect of pH on development and polyspermy in *Arbacia punctulata*. Open circles, development; solid circles, polyspermy.

exactly. The sea water buffers were broken down by the addition of 23 cc. 0.1N HCl/liter of sea water, and the water was allowed to stand overnight in a shallow dish to permit escape of the excess CO_2. The pH was adjusted by the addition of 0.1N NaOH, and checked with Clark and Lubs indicators. Eggs were inseminated in the solutions after 10 minutes exposure.

The results, as shown in Table XII, correspond well with those of Smith and Clowes for *Arbacia*, when allowance is made for the lighter

insemination. It is interesting to note that the percentage of polyspermy is perceptibly increased over the normal sea water control when the carbonate buffer system is broken down and the pH brought back to 8.2 by the addition of NaOH. As reported by Smith and Clowes, the greatest incidence of polyspermy occurs at about neutrality. As they are plotted in Fig. 4, the data show a somewhat wider range over which polyspermy occurs, as well as a more gradual rise both to 100 per cent fertilization and to the maximum amount of polyspermy.

Discussion

As in the case of activation of egg cells, the range of effective agents for the production of polyspermic fertilization in sea urchin eggs is exceedingly wide and bewilderingly contradictory. Simple explanations are immediately ruled out by data proving the efficacy of an agent which increases the rate of metabolism—heat, and one which decreases it—cold; of an excitant—strychnine, and a depressant—alcohol; of a substance which raises permeability—Na, and a substance which lowers it—Mg.

A list of the agents or conditions which have been shown to produce polyspermy is given here, to indicate the wide field which they cover.

Agent	Investigator
Physico-chemical	
Heat	Hertwig, O. and R., 1887
Cold	Hertwig, O., 1890
	Bury, J., 1913
Acid sea water (pH 7.0–7.2)	Smith and Clowes, 1924
	Rose, M., 1931
Egg and sperm extracts	Sampson, M., 1926
	Godlewski, E., 1934
Common cations	This paper
Alkaloids	Hertwig, O. and R., 1887
Fat solvents	
CH_3Cl	Hertwig, O. and R., 1887
Ether, ethyl alcohol, ethyl urethane	This paper
Chloral hydrate	Hertwig, O. and R., 1887
Physiological	
Mechanical injury	Hertwig, O. and R., 1887
Age	Hertwig, O. and R., 1887
Immaturity	Fol, H., 1877
Overripeness	Fol, H., 1877
Poor condition of parent animals	Fol, H., 1877
	Hertwig, O., 1890
Overinsemination	Gemmill, J. J., 1900

It is very probable that further work will show still more diverse substances to be equally effective, since the effort to extend the list in the past has been largely directed by one theory—that polyspermy is caused by substances or conditions which tend to slow up the speed of some cortical reaction. When this point of view is disregarded, other agents can be added to the list which may in time contribute to a generalized concept and to further analysis of the steps in the fertilization reaction.

One fact which is of undoubted importance in connection with an understanding of experimentally induced polyspermy is that in very many cases a direct correlation can be observed between the severity of the treatment—time of exposure × concentration of reagent—and, at first, the number of eggs which is polyspermic and later, when they are all affected, the number of sperm which enter each egg. The significance of this point lies in the fact that it definitely disproves the existence of an all-or-none type of mechanism, and indicates rather a reaction which may be retarded or accelerated by the treatment.

From the fact that the shortest possible time of exposure to most of the reagents gave at least half as much polyspermy as much longer exposures, it is evident that the reaction which is responsible for the appearance of polyspermy is initiated very rapidly. The complete reversal on return to sea water which was observed with optimum treatment (e.g., 0.2 per cent chloral hydrate) again emphasizes the rapidity of the process, besides indicating its reversibility. It is necessary to point out in this connection that the interval between introduction of the eggs into a given solution containing sperm and their fertilization consists in little more than the time which it takes for the sperm to penetrate the egg jelly, since the sperm are well dispersed and must reach the eggs as soon as they enter the solution.

The uniformly short time of exposure which is effective with all reagents in spite of the fact that the living cell membrane intervenes between the egg protoplasm and its environment, and in spite of the very different rates of penetration into cells of the various substances involved, would lead to the supposition that whatever mechanism is affected lies at or immediately beneath the cell membrane. This is, of course, the location which would be postulated for a reaction that is concerned with stimuli entering the cell from the outside, and which is deranged by treatment that leaves other cell functions apparently normal.

The reversibility after moderate, and the irreversibility after severe treatment with chemical agents is an important characteristic of

experimental polyspermy, and one which will materially assist in defining the reaction by excluding many types of effects which are known to be consistently irreversible, and by affording an index of severity of treatment. The anomalous case of quinine SO_4, which doubles its effectiveness on return to sea water instead of reversing to normal, provides interesting material for speculation, but it cannot be considered to invalidate the other results since so little is known of the actual mode of action of this substance.

It is certainly apparent that the information at hand is insufficient basis for any final conclusion concerning the mechanism of monospermy. However, as working hypotheses, some of the possibilities which these data suggest can be used to advantage in a further attack on the problem. For example, it is known that Na, K, Mg (Heilbrunn and Daugherty, 1932), ether (Heilbrunn, 1934), and cold (Thornton, 1935) cause a change in the viscosity of cortical protoplasm in the direction of liquefaction. All these agents have been shown to be more or less effective agents for causing polyspermy. In view of the fact that a marked gelation of the sea urchin egg cortex occurs at fertilization (surface pigment granules which are easily moved by centrifugal force before fertilization cannot be dislodged immediately afterward), it is possible that interference with this gelation would disturb the supernumerary sperm-excluding mechanism to the extent of producing polyspermy.

Another observable change in the physico-chemical state of the sea urchin egg cortex which is closely connected in some way with the fertilization reaction is the shift in color of the egg surface with dark-field illumination, as reported by Runnström (1928). The color has been shown to be caused by the lipoid constituents of the outer membrane. Dark-field observations of treated eggs with special attention to the coloration of the lipoid layer would determine whether or not the change in the coloration of the lipoid layer seen by Runnström is important in connection with monospermy.

This study, then, has shown that the problem of polyspermy can be attacked by measures which will yield quantitative results, and it is possible that data from this source may constitute a significant addition to the knowledge of the fertilization reaction.

Summary

1. Experimental study of pathological polyspermy is discussed as a method for ascertaining the dynamic characteristics of the fertilization reaction and especially of monospermy.

2. A general procedure is described for treatment of unfertilized

eggs, standardization of sperm suspensions, and determination of percentages of polyspermic fertilizations.

3. Comparable overinsemination of the eggs of *Arbacia punctulata*, *Asterias forbesii*, *Cumingia tellinoides* and *Cerebratulus lacteus* shows sharply decreasing resistance, in the listed order, to polyspermy caused by excess sperm.

4. Eggs of *Asterias forbesii* become susceptible to polyspermic fertilization after standing in sea water less than two hours. Eggs of *Arbacia* show little diminution in their normal resistance to polyspermy until after sixteen hours in sea water.

5. Unfertilized *Arbacia* eggs were exposed to dilute solutions of chloral hydrate, cocaine hydrochloride, nicotine, morphine sulphate, strychnine sulphate and quinine sulphate. The effectiveness of these substances in inducing polyspermy is discussed and compared. The results for *Arbacia punctulata* are found to be qualitatively similar to those obtained by O. and R. Hertwig (1887) with *Strongylocentrotus lividus*.

6. Qualitative experiments testing the effect of fat solvents on unfertilized *Arbacia* eggs showed a fundamental similarity between the effects of anæsthetic concentrations of ethyl alcohol, ether, chloroform and ethyl urethane. These substances have a narrow range of high effectiveness near the point at which the cell cytolyzes.

7. Studies of the effect of the four common cations of sea water in causing polyspermic fertilization of *Arbacia* eggs showed that, when unfertilized eggs are washed in isotonic solutions of the chlorides and inseminated on return to sea water, the ions are effective in the order $Na > K > Ca > Mg$. On the other hand, insemination in isotonic chloride-sea water mixtures gave the result, $Mg \gg Na, K, Ca$, with the maximum percentage for $Mg = 71$, for Na, K and Ca = ca. 5.

8. Exposure of unfertilized *Arbacia* eggs to acidified sea water over the pH range from 5.8 to 8.2 gave a maximum of polyspermy at pH 7.0. This result agrees with the work of Smith and Clowes (1924).

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THE EFFECT OF TEMPERATURE ON OVIPOSITION IN DROSOPHILA MELANOGASTER

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A study of the effect of temperature on oviposition rate in *Drosophila melanogaster* was undertaken with the hope that the egg-laying mechanism might offer suitable material for the study of growth problems in the formation of the mature egg, a process which is free from the complications of differentiation.

METHODS

To eliminate genetic differences sibling F_1 females from single-pair crosses between inbred strains of vestigial and Oregon-R wild type flies were used for the egg-laying. These inbred lines had been maintained by single-pair matings during four generations for the vestigial line, and seven generations for the Oregon-R line. Wings of the females were clipped to prevent accidental loss of flies.

Single females were isolated in shell-vials within 24 hours after eclosion from the pupa-case, and mated with one or two males. Males dying during the course of the experiment were immediately replaced.

Eggs were collected on paper ice-cream spoons (previously impregnated with bees-wax to prevent moisture absorption) containing 2 cc. of a banana-molasses food medium (100 cc. water, 20 grams banana, 5 cc. molasses, 2 grams agar) yeasted with banana-cultured yeast. Egg-laying was confined to the food surface. In cases where moisture collected on the walls of the vials, examination showed that no eggs were laid in this moisture.

Females were allowed to lay eggs for a 24-hour period on any one spoon, a record was made of the number of eggs laid, and a fresh spoon supplied for the next 24-hour period.

To detect any differences between females ovipositing at a temperature other than that at which they were bred, and those ovipositing at the respective temperatures at which they were bred, the experiments were arranged as follows:

(1) Females were bred at 16° C., 20° C., 25° C., and 28° C., and allowed to oviposit at the same temperatures.

TABLE I

Averaged data for the total eggs laid up to the end of an elapsed time. The number of females averaged is indicated by the numbers between parentheses. The group numbers at the column-heads correspond to the numbers on the curves of Fig. 1.

Days elapsed	Total eggs laid							
	All females bred at 25° C. Ovipositing at temperatures indicated					Females ovipositing at the respective temperatures at which they were bred		
	2. (5) 16° C.	4. (5) 20° C.	5. (7) 25° C.	6. (5) 25° C.	8. (4) 28° C.	1. (5) 16° C.	3. (6) 20° C	7. (6) 28° C.
1	0	0	3	8	15	0	1	9
3				80				
4		28						
5	18						24	262
6			165		464	73		
7		106						
8				478				
10	48						121	717
11			353		943			
12		221				181		
13				763				
15	119						262	1054
16			554		1258	259		
17		367						
18				977				
20	230						432	1305
21			780		1507	383		
22		529						
23				1157				
25	318						587	1477
26			1076			463		
27		711			1634			
30	411						815	1519
31			1270			511		
32		886						
35	513						1005	
36			1455			574		
37		1068						
40	591						1121	1519
41			1594			686		
42		1206						
45	629						1206	
46			1708			741		
47		1305						
50	679						1406	
51						777		
52		1422						
55	770						1550	
56						810		
57		1528						
60	816						1646	
62		1790						
64		1834						
65	856						1700	
70	875						1775	
73	901							

(2) Females were bred at 25° C., and transferred to 16° C., 20° C., and 28° C., at which temperatures their eggs were laid.

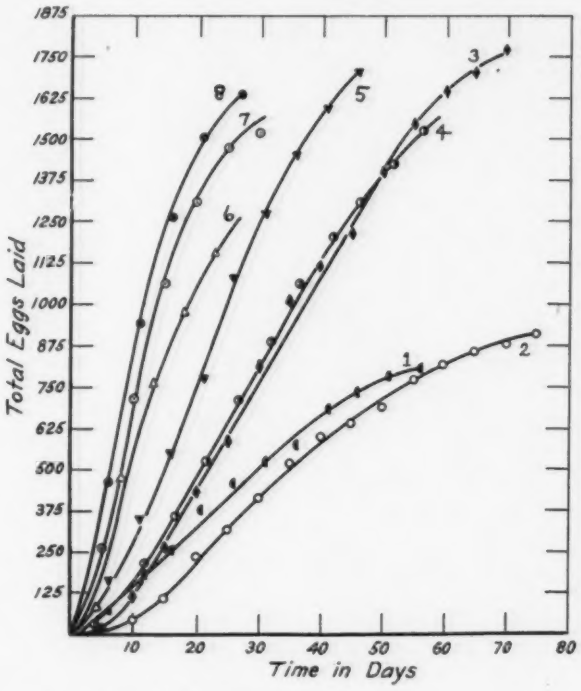

FIG. 1. Plots of the data of Table I.

1. Females bred at 16° C., and ovipositing at 16° C.
2. Females bred at 25° C., and ovipositing at 16° C.
3. Females bred at 20° C., and ovipositing at 20° C.
4. Females bred at 25° C., and ovipositing at 20° C.
5. Females bred at 25° C., and ovipositing at 25° C.
6. Females bred at 25° C., and ovipositing at 25° C.
7. Females bred at 28° C., and ovipositing at 28° C.
8. Females bred at 25° C., and ovipositing at 28° C.

Females of Curves 2, 4 and 5 are siblings. Females of Curves 6 and 8 are siblings. The counts for Curve 6 were discontinued before the end of egg-laying because of an accidental rise in the temperature of the 25° incubator. The females of Curves 1 and 2 were not followed through until the natural end of egg-laying.

The temperature for each incubator varied $\pm$ 0.5° C. from the intended value.

Results

Since the rate of egg production in the fruit-fly changes rapidly with time (Shapiro, 1932), a convenient comparison of the temperature effect on egg production is obtained from a plot of the total eggs laid up to a given time. Table I gives the results for each temperature of development and egg-laying. In the table, averages of from 4–7 females (as indicated) are used. Averaging is justified because prac-

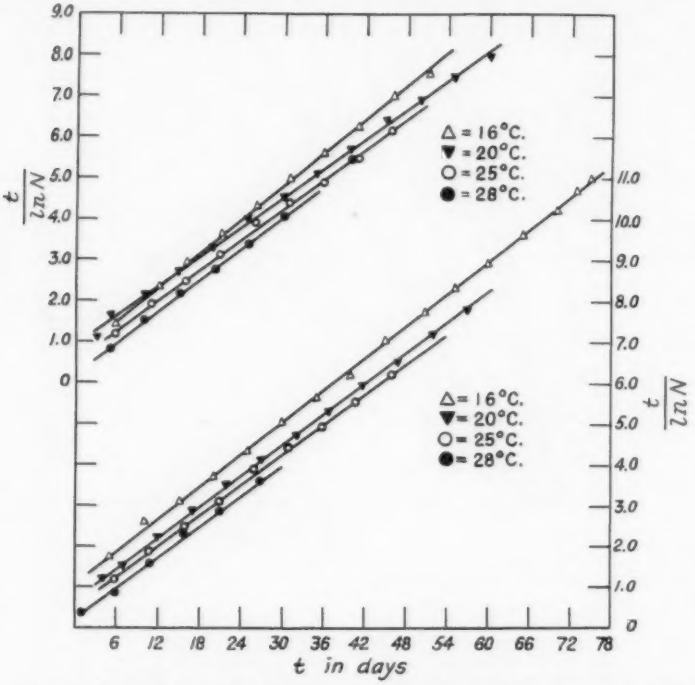

FIG. 2. Plots of the data presented in Table I, using Shapiro's equation of $t/lnN = at + b$. The curves in the upper half of the figure are for females ovipositing at the same temperatures at which they were bred. The curves in the lower half of the figure are for females that were bred at 25° C. and transferred to the other temperatures to oviposit.

tically identical curves are obtained for each female of any one experiment.

The data of Table I are shown graphically in Fig. 1. Although all the curves are sigmoid in shape, their central portions may reasonably be considered linear. By disregarding the curved initial and final

portions, which in all the experiments (except perhaps at 16° C.) represent very small fractions of the total number of eggs laid, we may use the slopes of the linear portions of the curves as the characteristic rates of egg-laying at the various temperatures.

The curves in Fig. 1 are grouped in pairs, representing the two different experimental set-ups at each temperature (*cf.* under "Methods" above). The two curves at each temperature can be seen not to differ significantly from each other, indicating that the rate of egg-laying is independent of the temperature at which the females were bred, and depends only on the one at which the eggs are being laid.

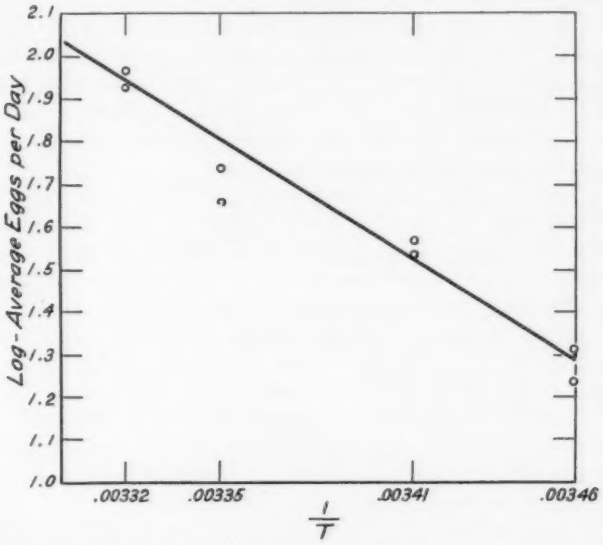

FIG. 3. Arrhenius plot of the data presented in Fig. 1. The number of eggs laid per day is calculated from the straight-line portions of the curves of Fig. 1.

The variation between the two curves (5 and 6, Fig. 1) for 25° C. is probably due to the fact that the two sets of females have different pedigrees.

Shapiro (*loc. cit.*) had shown that when the ratio t/lnN, where t is the number of elapsed days of egg-laying, and N the number of eggs laid at the end of time t, is plotted against t, a straight line is obtained. Shapiro expressed this linear relationship by the equation $t/lnN = at + b$, where a and b are constants. The same plots of t/lnN as ordinate against t as abscissa were made with our data, and

the curves are shown in Fig. 2. Straight lines are obtained at each temperature, indicating that whatever relationship the equation represents remains constant when the temperature is varied.

Figure 3 represents a plot of the logarithm of the rate of egg-laying against the reciprocal of the absolute temperature. The rates of egg-laying were obtained from the slopes of the linear portions of the curves given in Fig. 1 and are expressed as eggs per day. The straight line joining the points in Fig. 3 seems to show that the Arrhenius equation applies in this case, and that the rate of egg-laying is a function of temperature in the same manner as are the rates of many chemical reactions.

Conclusions and Discussion

The regularity of the data at the different temperatures indicates that the mechanism of oviposition in *Drosophila melanogaster* may be useful for further study. The applicability of both the Shapiro and the Arrhenius equations further indicates that the underlying factors of oviposition are uniform in their action.

The rate of oviposition has been used by Dobzhansky (1935) with *D. pseudoöbscura* as an indicator of differences between geographic stocks. It has been used to measure physiological effects of genes (Alpatov, 1932) on the assumption that different Mendelian characters affect differently the developmental period of the fly.

The question arises as to what determines the total egg production throughout the life of the fly. Does it start out with a fixed amount of egg-material available for egg production, the rate of egg-laying being a measure of the rate of consumption of this material, or does the amount of available egg-material vary with the temperature under which the flies are bred, the rate of oviposition being a measure of the operation of some other factor or factors?

The data at hand are not adequate to answer the question specifically. However, examination of Fig. 1 shows that although the total numbers of eggs laid by the females at 20° C., 25° C., and 28° C., tend to approach the same asymptote, there is nevertheless a small likelihood that the curves for 16° C. will reach the asymptotes of the curves for the flies laying at the higher temperatures. Furthermore, the curves for flies bred at 25° C. and 16° C. respectively, and both laying eggs at 16° C., show identical trends as regards rate. The latter fact would indicate that the available egg-material does not enter as a factor in this case. At the higher temperatures, however, the tendency for all the curves to approach the same asymptote seems to show that the available egg-material may be a limiting factor for these flies.

The egg-laying process as a whole must involve at least four components: first, the feeding habits of the females; second, the production of secondary oöcytes; third, the accumulation of stored materials in the growing egg; and fourth, the physiological reaction of oviposition. It may be possible to separate these processes and study each of them independently.

Summary

For any given temperature of egg-laying, there are no significant differences in rate of oviposition between females that have been bred at that temperature, and those that have been bred at 25° C.

The rate of egg-laying (as measured by the number of eggs laid per day) starts low, rises to a maximum, and decreases.

The rate for the middle period of egg-laying is practically constant.

The rate of egg-laying is related to temperature according to the Arrhenius equation.

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THE EFFECTS OF X-RADIATION ON THE REGENERATION OF THE HYDROID, PENNARIA TIARELLA

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INTRODUCTION

Investigations of the past decade have demonstrated that X-radiation is a valuable tool in studying the process of regeneration in various animal groups. By means of X-radiation, abnormal conditions have been produced in the tissues and cells of normally regenerating structures which result in their failure to regenerate and in this way light has been thrown on their probable function in the normal process of regeneration. Much work of this type has been done among certain phyla of the invertebrates. Among these investigations may be mentioned the work of Curtis (1928) on *Planaria;* Stone (1932 and 1933) on *Tubifex* and Turner (1934 and 1935) on *Lumbriculus.*

Only two papers have reported studies on the effects of X-radiation on regeneration processes in the cœlenterates. Curtis and Ritter (1927) state that hydranths of *Tubularia crocea* fail to regenerate after exposure to X-radiation and Zawarzin (1929) and Strelin (1929) report that regeneration in *Pelmatohydra oligactis* is inhibited by X-radiation. Strelin (1929) has made histological studies of non-regenerating X-radiated forms and states that the failure to regenerate is due to the destruction of the interstitial cells by the X-radiation.

In the present investigation studies have been made to determine (1) the effects of X-radiation on the regeneration of amputated hydranths of *Pennaria;* (2) the relative sensitivity to X-radiation of hydroid tissues as determined by the minimal dosage of X-radiation necessary to inhibit regeneration and (3) the effects of X-radiation on the regeneration of a colony of hydranths in which a portion of the colony has been exposed to X-radiation with the remaining connected portion of the colony screened from the radiation by a lead plate. The observations on these studies will be described here. It is proposed to make a histological study of the materials collected in these experiments with the hope that they can be reported at a later date.

MATERIALS AND METHODS

Colonies of the hydroid, *Pennaria tiarella,* were used in all the experiments. This form is found growing profusely on the brown alga,

Fucus, in the vicinity of Woods Hole, Massachusetts, during the months of July and August. Fresh material was brought into the laboratory daily and only day-old material was used in the experiments. The hydranths were removed from young branches of the colony under a dissecting microscope. After radiation the branches were suspended by means of fine cotton threads in dishes of running sea water for the duration of the experiment.

The source of radiation was a Coolidge broad focus tube. The factors governing the radiations were as follows: 120 Kv.P., 5 ma., distance from target to material, 19.5 cm. The radiation was unfiltered. The output of the tube as determined by a standard ionization tube was 750 Roentgen units per minute. The only variable factor was the length of exposure and this will be mentioned in connection with the various experiments.

Normal Regeneration of Hydranths

The process of hydranth regeneration in *Pennaria* has been discussed in some detail by Gast and Godlewski (1903) and Hargitt (1904). In addition to experimental work, these investigators report histological studies on the normal regeneration of hydranths of *Pennaria* and the reader is referred to these papers for a more detailed account of the process than can be covered in this paper.

Amputated hydranths of *Pennaria* are ordinarily regenerated in 36 to 48 hours when the branches are kept suspended in running sea water at a temperature of around 22° C. Early in the investigation it was noted that *Pennaria* tended toward heteromorphic regeneration. If the pieces under observation were simply laid flat on the bottom of the dish, the cœnosarc, on emergence from the perisarc, came in contact with the dish and in most cases holdfasts were regenerated rather than hydranths. Hydranths were regenerated only at the free ends. To avoid this contact reaction the branches under observation were suspended in dishes of running sea water by fine cotton threads. In this manner all the cut ends were bathed by sea water and only hydranths were regenerated, even at the basal end of the stem. As stated above, new hydranths begin to appear 36 to 48 hours after amputation. Not all the regenerating hydranths appear in so short a time, but new hydranths continue to form on cut stems of the branch for a period of days. Colonies, on the average, were found to regenerate about 70 per cent of their lost hydranths.

If at the end of several days these regenerated hydranths are amputated, a second regeneration takes place in about the same period as that mentioned above. This process of re-amputation has

been repeated for three or four successive times. New hydranths continue to regenerate.

Morphologically, the process of hydranth regeneration appears to be fairly simple. Wound closure takes place in six hours, or less, and appears to be due to an approximation of the cut edges of the cœnosarc. Regeneration processes then set in and 12 hours after amputation the first indication of a regenerating hydranth is the formation of a knob-like protuberance by the cœnosarc. This protuberance is covered by a thin layer of perisarc, which is later ruptured when the new hydranth begins to take form. This bud of cœnosarc continues to grow and tentacles appear about 24 hours after amputation as small evaginated buds on the body of the hydranth. The tentacles assume their normal length by new growth. The proboscis appears to form as an evagination from the distal end of the bud, and late in the process of regeneration the mouth opening breaks through. Regeneration is complete 36 to 48 hours after amputation.

X-radiated Branches of Pennaria

The following experiment was carried out to determine the effects of X-radiation on hydranth regeneration in *Pennaria*, and to obtain data as to the relative sensitivity of this hydroid to X-radiation, as compared with various other invertebrates on which similar studies have been made.

Small branches of *Pennaria*, possessing, on the average, twenty to twenty-five hydranths were used in all experiments. The hydranths were removed under a dissecting microscope and immediately radiated in small dishes of sea water. In the preliminary experiments dosages of 750, 1500, 3750, 7500, 10,500, 15,000 and 22,500 Roentgen units were given to various branches. The results of these experiments demonstrated that dosages of 10,500 Roentgen units, or larger, completely inhibited the regeneration of new hydranths. Dosages of less than 10,500 *r* retarded the regeneration of hydranths but were not sufficient to inhibit their formation completely. In all the later experiments, therefore, the minimal inhibiting dosage of 10,500 *r* was used.

It was found that X-radiation did not cause an immediate check on regeneration processes in radiated branches, but considerable growth of fresh tissue took place during the first twelve to eighteen hours after radiation. As a result, there was the formation of a rounded bud of fresh tissue at the cut surface of the branch, but these buds were never able to differentiate into new hydranths. Many of these radiated branches were kept under observation from 9 to 12 days after amputation without showing any evidences of differ-

entiating into new hydranths. In some cases there was a continued growth of this new tissue, so that long stolon-like structures, several millimeters in length, were formed.

Reference to section *A* of Fig. 1 will show the extent of regeneration in radiated branches. Here it will be seen that there is a considerable

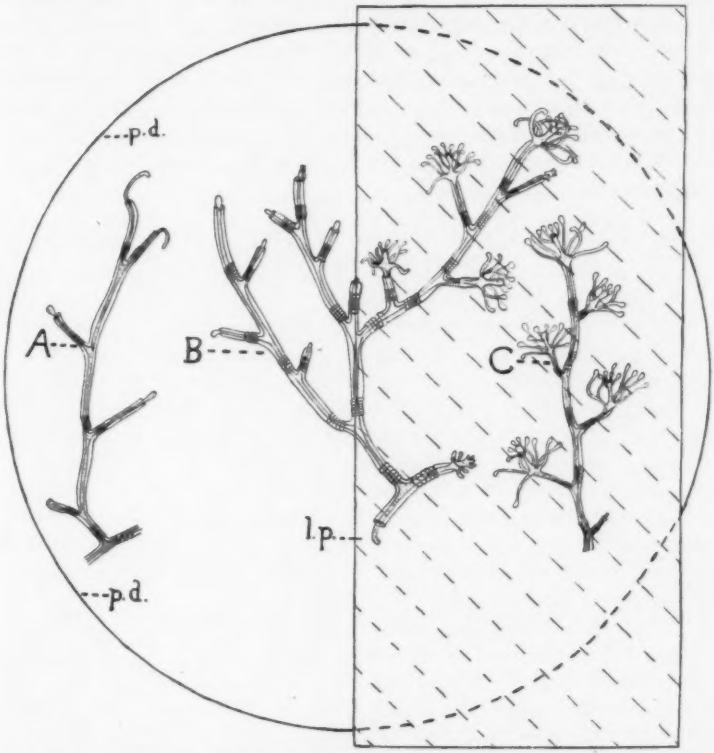

FIG. 1. Diagram of apparatus used for the radiation of a portion of a *Pennaria* colony. Colony *A* is a radiated control; colony *C* a screened control; while only a portion of colony *B* has been exposed to radiation as explained in text. *l.p.*, lead plate (cross-hatched); *p.d.*, edge of Petri dish.

proliferation of fresh tissue beyond the point of amputation but this tissue appears to be powerless to differentiate into new hydranths.

If these non-regenerating branches of X-radiated *Pennaria* be re-amputated 4 to 6 days after the first amputation, the above process is repeated. A proliferation of fresh tissue takes place but there is never a formation of new hydranths.

Other experiments were carried out with branches of *Pennaria* in which radiation was made four days prior to the amputation of the hydranths. In these experiments the results were essentially the same as described above. A small proliferation of fresh tissue takes place but in no cases were hydranths ever formed.

The results of these experiments were clear-cut. Hydranths of *Pennaria tiarella* fail to regenerate when exposed to dosages of X-radiation of 10,500 Roentgen units, or more. Smaller dosages retard the regeneration of hydranths, but fail to check the process completely. The primary effect here appears to be on cellular differentiation rather than on cellular proliferation. In many cases, an adequate volume of fresh tissue is regenerated, but this tissue appears powerless to form a new hydranth.

Experiments in which a Portion of the Colony was Shielded from the Radiation

In *Pennaria*, as in other hydroids, the individual hydranths are connected by a common cœnosarc and share a common circulation by means of a cœlenteron. The question arises as to what will be the effect of X-radiation on the regenerative abilities of a colony if a portion of the colony is exposed to the radiation while the remaining portion of the colony is shielded from the radiation by a lead plate. Will toxic substances or so-called "necrohormones" be formed in the exposed portion of the colony and then pass over to retard or inhibit hydranth regeneration in the screened region? On the other hand, will the protected members of the colony exert any sort of influence on the non-regenerating X-radiated portion of the colony so as to promote regeneration in this region?

To test these possibilities the following experiment was carried out. A small Petri dish was fitted with a one-quarter-inch lead plate so that one-half of the surface of the dish was covered and the other half exposed to the radiation as shown in Fig. 1. The inner surface of the plate was bent into the shape of a flange extending to the bottom of the dish so as to prevent a scattering of the radiation under the lead plate. A piece of the central stem of a *Pennaria* colony (Fig. 1, colony *B*) with two lateral branches of approximately equal size, and from which all the hydranths had been amputated, was placed in the dish in a manner such that one branch was exposed to the radiation while the remaining connected portion was protected by the lead plate. One completely exposed colony (Fig. 1, colony *A*) and one completely screened colony (Fig. 1, colony *C*) were used as controls. The whole set-up, as described above, was given a single exposure to X-radiation of 10,500 Roentgen units.

Briefly stated, the results were as follows: The colony exposed to the radiation (colony *A*, Fig. 1) failed to regenerate its lost hydranths, even after six to eight days after radiation. The colony completely screened from the radiation (colony *C*, Fig. 1) regenerated its lost hydranths in 48 hours while the partially exposed colony (colony *B*, Fig. 1) behaved as follows: The portion of the colony protected from the radiation by the lead screen regenerated its lost hydranths in 48 hours, as rapidly and as completely as its control (colony *C*), while the exposed portion of the same colony failed to regenerate, even as long as eight days after amputation. The exposed portion behaved in all respects like the completely exposed control (colony *A*). This experiment was repeated several times with the same results.

The results obtained here demonstrate that, for the duration of the experiment, the effects of the X-radiation are purely local. The fact that screened members of the colony, although they are connected directly with non-regenerating X-radiated members of the same colony, regenerate at a normal rate tends to discount the idea that anything like a toxic material or "necrohormone" is being formed in the radiated portion of the colony which may be spread throughout the entire colony. In a like manner, there is no evidence that any regeneration-promoting substance or material is passing from the unradiated portion of the colony over to the non-regenerating branches of the radiated side. They act as if they were independent of the unradiated tissue.

Discussion

Considerable evidence is being accumulated to show that one of the chief biological effects of X-radiation is an effect on cellular differentiation. Histological studies on non-regenerating limbs of X-radiated *Amblystoma* larvæ made by Butler (1933) and Puckett (1936) show that one of the chief effects of the radiation is to prevent the differentiation of the cells of the limb stump into new limb structures. Likewise, Henshaw (1935), in his studies on the effects of X-radiation on the development of the eggs of *Drosophila*, has found that the lethal effect of X-radiation on *Drosophila* eggs is not the result of an inhibition of cell divisions, but is due to the fact that the new cells that are formed are powerless to differentiate into embryonic structures. Death results, not from a cessation of growth processes, but as a result of the loss of the powers of directive growth and organization.

Although no histological studies have been made on non-regenerating X-radiated branches of *Pennaria*, the effect here appears to be primarily on differentiation processes. In practically all cases of radiated branches of *Pennaria*, a mass of new tissue sufficient to form

a new hydranth was regenerated and, in many cases, fresh tissue of several times the volume of a hydranth was formed. However, this tissue appeared to be unable to differentiate into hydranths. It seems clear that the effect of X-radiation in this case is not primarily an inhibition of growth but rather an inhibition of the ability to differentiate new structures.

The observations made here also demonstrate that the effect of X-radiation is a local one on radiated tissue. The fact that exposed portions of a colony of hydranths fail to regenerate after exposure to X-radiation, while connected portions of the same colony, screened from the radiation, regenerate at a normal rate supports the statement above. This evidence seems to show that the tissue fluids of the colony are little affected by the radiation. The various members of the hydroid colony are connected by a common cœnosarc and have a common circulation in the nature of a cœlenteron. If the tissue fluids exposed to the radiation were affected they should, in some way, affect the regeneration of the protected hydranths. This does not appear to be the case.

This latter observation agrees with and substantiates the observations of Butler (1935) in his studies on the effects of local radiations on the regeneration of the *Amblystoma* limb. He found, to mention a single experiment, that if the body of the larva be radiated with the limb screened from the radiation, then regeneration was not interfered with. On the other hand, if the body of the larva was screened and the limb exposed to the radiation, then the limb failed to regenerate if amputated. This, and a series of other experiments, showed conclusively that the effect of X-radiation was a local one on the cells of the limb, rather than on the general body fluids such as the blood or lymph. The hydroid experiments appear to confirm his belief in a purely local effect of X-radiation.

Summary

Regeneration of amputated hydranths of the hydroid, *Pennaria tiarella*, has been prevented by exposure to X-radiation.

The minimal dosage of X-radiation necessary to inhibit hydranth regeneration has been determined.

The effect of X-radiation appears to be primarily on cellular differentiation, rather than on cellular proliferation. In all cases an appreciable amount of fresh tissue regenerated, but this tissue appeared powerless to differentiate into new hydranths.

X-radiation of one portion of a single colony of *Pennaria* with the remaining connected portion of the same colony protected from the

radiation by a lead screen, indicates that the effects of radiation are local. Exposed portions of the colony failed to regenerate new hydranths, while protected portions regenerated new hydranths at a normal rate.

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THE QUANTITATIVE DETERMINATION OF MITOTIC ELONGATION

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This is the first of a series of investigations dealing with a specific phase of cell division, the elongation which occurs when a cell divides. The normal elongation of the cell may be defined as follows: the spontaneous extension that ensues upon the increase in length of the diaster, and which is climaxed by the cleavage of the cell. Measurements have been made of the magnitude of this extension, and time-elongation relationships established. The present paper is concerned only with the normal process; future experiments have been planned to test the effects of various physical and chemical factors. Some of these experiments have been partially completed.

Because the process of mitotic elongation is essentially of a kinetic nature, involving changes in cell structure with respect to time, it can be studied best in the living material. It was not feasible to employ the methods of fixation and staining in order to determine the morphological changes. Nevertheless, an attempt was made to insure an adequate correlation between structure and function. This was done by selecting material that would show the cell structures most clearly in the living animal.

In this respect the egg of the common sea-urchin, *Arbacia punctulata*, possesses certain advantages. The achromatic figure (diaster) is well-defined in the living material and indicates distinctly the future axis of elongation. Since the egg is not always exactly spherical prior to the period of elongation and cleavage, the clear bipolar spindle serves to indicate, and allows one to measure, that particular diameter of the egg that will coincide with the future axis of elongation. Another advantage is to be found in the fact that the two blastomeres resulting from the first cleavage never separate entirely. This precludes any errors arising from a shifting or twisting of the blastomeres that might otherwise occur.

[1] Opportunity is here taken to thank Dr. L. V. Heilbrunn for his valuable assistance and criticism during the course of this investigation.

The manner of procedure is exemplified by Experiment *C* of Series *A*. The particular egg used in this experiment was fertilized at 3:02 P.M., and the first measurements of the diameter were made at 3:45 P.M.—just prior to the onset of the elongation. The average value for the egg diameter was 74.9μ. Successive elongations (column 7—as computed by subtracting the initial from the final readings) were measured with a filar micrometer, and the "times" (column 4) corresponding to the readings were "tapped out" on a key connected

SERIES *A*

Mitotic elongation of *Arbacia* eggs with fertilization membranes.

7/31/35 Fert.	Diameter *ab* of egg (scale div.) add 300		*T* sec.	Remarks	Original diameter	Successive increments	Max. Δr	Increase
	a	*b*						
								per cent
Exp. *C*			0_t=3:45	R.T. = 23.6° C.	325.5		33.5	10.3
3:02 P.M.	22.5	46.5	P.M.	tight fert.				
	21.5	48.0		membrane				
	20.5	46.5			(=74.9 μ)		(=7.7 μ)	
	20.5	47.5						
	19.5	44.0						
	19.0	46.5	0	*		27.5		
	18.0	52.5	30			34.5		
	21.0	60.5	60			39.5		
	25.0	73.5	102	touching		48.5		
	23.0	79.5	132	fert. mem.		56.5		
	22.0	81.0	159			59.0		
	20.5	79.5	183			59.0		
	20.5	78.0	225			57.5		
	22.5	73.5	261			51.0		
	25.0	69.0	306			44.0		
	24.5	65.0	342			40.5		

One scale division = 0.23 μ.
* First appearance of the elongation.

with a kymograph on which timing intervals of 6 seconds were automatically being recorded. The maximum elongation is seen to be 7.7μ, an increase of 10.3 per cent.

The measurements for 27 cases are presented in Table I. The average diameter of these 27 eggs before elongation is 73.5μ, with a probable error of $\pm$ 0.224μ. The "probable error" has the usual definition, $R = \pm 0.6745\sqrt{\frac{\Sigma(V^2)}{n(n-1)}}$. The table shows that there is no correlation between the diameter of the spherical egg, and the

maximum diameter that it will reach during the elongation. In other words, given two eggs of different diameters, nothing can be predicted concerning which of them will attain the greater diameter during division. The average length of the major axis of the eggs when in the state of maximum elongation is $82.0 \pm 0.332\mu$.

The experimental temperatures (see Tables I and II) ranged from 22.2° C. to 26.0° C., a variation of 3.8° C. The variation for any one experiment was always less than 1.0° C. Some preliminary experiments conducted at low temperatures (7° C.–10° C.) indicate that this process may have a high temperature coefficient. It is indeed unfortunate that the temperature could not be more uniformly con-

TABLE I

Mitotic elongation of *Arbacia* eggs with fertilization membranes.

Temperature	Original diameter	Max.	Δx	Increase	Temperature	Original diameter	Max.	Δx	Increase
° C.	μ	μ	μ	*per cent*	° C.	μ	μ	μ	*per cent*
23.8	73.4	81.5	8.1	11.0		71.4	77.5	6.1	8.5
24.0	75.9	84.0	8.1	10.6	23.0	71.8	81.8	10.0	13.9
23.8	71.0	79.4	8.4	11.8		72.7	86.0	13.3	18.1
23.5	72.0	81.3	9.3	12.9	22.2	72.8	81.0	8.2	11.3
23.5	74.8	83.1	8.3	11.1	23.0	78.5	87.5	9.0	11.5
23.0	73.0	82.7	9.7	13.0	23.0	74.1	82.6	8.5	11.5
23.0	74.8	79.7	4.9	6.6	22.7	72.5	81.9	9.4	12.9
23.0	71.8	80.8	9.0	12.5	22.4	76.0	84.4	8.4	11.3
23.0	74.4	80.5	6.1	8.2	23.0	74.1	87.1	13.0	17.5
23.0	74.8	80.2	5.4	7.1	22.5	70.8	76.9	6.1	8.6
23.0	72.2	78.8	6.6	9.1	23.6	74.9	82.6	7.7	10.3
	72.8	83.3	10.5	14.4	24.4	72.5	79.7	7.2	9.9
23.7	74.1	81.9	7.8	10.5	24.0	73.4	83.2	9.8	11.9
	72.8	83.6	10.8	14.8					

At R.T. of 22.2° C.–24.4° C., the mitotic elongation is 11.5 per cent; or, an egg 74.0 μ in diameter elongates to 82.5 μ.

trolled; yet, in view of the large biological variation, it seems certain that the resulting source of error is quite small.

If now, one plots "times" against "elongations" (Fig. 1), the result is two classes of curves: *A* and *B*. Type *A* is given only by those eggs whose fertilization membranes "stand off" from the egg surface. Type *B* is characterized by two maxima—due to the fact that the tight fertilization membrane impedes the elongation for a time. Cases falling in type *B* were the more numerous of the two. Indeed, the slopes of the curves, even within any one type, were quite divergent. The explanation of this is twofold: (1) the resistance of the fertilization membrane, and its distance from the egg are very

variable factors; and, (2) the difficulty—especially during the first stages of the extension—of knowing if one is measuring the maximum (or true) elongation, or a lesser diameter resulting from the fact that the plane of the spindle may not be exactly parallel to the plane of the slide. As cleavage progresses, the position of the furrow and the occurrence or nonoccurrence of slipping-over of the blastomeres provide criteria of the above source of error. Whenever such a slipping-over took place, the experiment was discarded. Moreover, rotational movements of the egg—occasioned by the limiting fertilization membrane—often proved annoying.

In view of these considerations, another set of observations has been compiled for eggs whose fertilization membranes had been re-

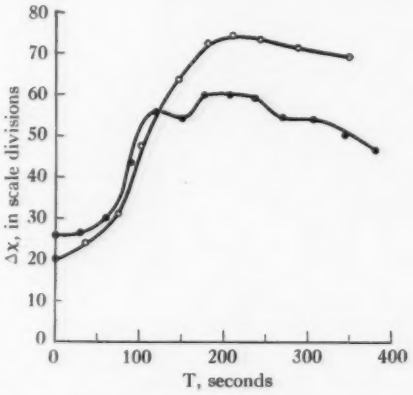

FIG. 1. Elongation of eggs having fertilization membranes. Open circles, Type *A*; solid circles, Type *B*.

moved by vigorous shaking some 1–4 minutes after insemination. No deformities resulted.

A typical record is shown in Experiment *C* of Series *B*; and Table II gives the measurements for 38 cases. The average diameter of the spherical eggs is 73.5 ± 0.171 μ, and following elongation the average maximum diameter of these eggs, whose fertilization membranes have been removed, is 102.3 ± 0.434 μ. Comparing the probable errors for all eggs, independent of the experimental conditions, one notes that the probable error for eggs under conditions of maximal activity (if one may so characterize the period of elongation) is much greater than the probable error for the diameters of the eggs while still spherical. That is to say, the variations found for the period of

elongation are not determined by the variations among the original diameters. Since Table II presents a greater number of cases than Table I (38 as against 27), it would be expected that the probable error of the former would be less than that of the latter. This is true for the measurements made on spherical eggs: the probable errors for the original diameters being ± 0.224 for Table I, and ± 0.171 for

SERIES *B*

Mitotic elongations of *Arbacia* eggs without fertilization membranes.

8/5/35 Fert.	Diameter *ab* of egg (scale div.) add 300		*T* sec.	Remarks	Original diameter	Successive increments	Max. Δ*x*	Increase
	a	*b*						
								per cent
Exp. *C*			0_i = 1:40	R.T. = 25.6° C.	330.0		133.5	40.5
12:58	92.0	21.0	P.M.	Removed				
P.M.	98.5	28.5		fert. mem.	(= 75.9 μ)		(= 30.7 μ)	
	81.0	14.0		1 : 01 P.M.				
	82.0	11.5						
	81.5	19.0	0	*		37.5		
	81.5	21.0	27			39.5		
	83.5	30.5	54			46.5		
	90.0	37.5	84			47.5		
	91.5	47.0	108			55.5		
	81.5	46.5	138			65.0		
	76.5	59.5	165			83.0		
	67.0	171.5	195			104.5		
	57.5	183.0	222			125.5		
	55.0	196.5	249			141.5		
	53.5	103.5	279			150.0		
	49.5	107.0	315			157.5		
	45.0	107.0	342			162.0		
	41.0	104.5	372			163.5		
	39.0	198.5	408			159.5		
	36.5	195.0	450			158.5		
	37.0	193.5	495			156.5		
	35.0	188.5	549			153.5		
	38.5	184.0	636			145.5		

One scale division = 0.23 μ.
* First appearance of the elongation.

Table II. On the other hand, the probable error for the eggs at maximum elongation is ± 0.332 for Table I, and ± 0.434 for Table II, results that could not be expected if the laws of chance alone were operating. The explanation of this apparent anomaly lies in the fertilization membrane factor; the rigidity of the membrane cuts down the upper limit, and therefore the range of possible variation.

of the elongation. As has been previously shown, not only does the fertilization membrane prevent the determination of time-elongation relationships, but it also prevents the determination of the true (maximum) extension.

In these experiments on eggs without fertilization membranes the time-elongation curves were all of one type: possessing a single maximum; and their slopes were much more in conformity with one another. The two curves in Fig. 2 show quite different maximum elongations,

TABLE II

Mitotic elongation of *Arbacia* eggs without fertilization membranes.

Temperature	Original diameter	Max.	Δr	Increase	Temperature	Original diameter	Max.	Δr	Increase
° C.	μ	μ	μ	*per cent*	° C.	μ	μ	μ	*per cent*
25.6	74.5	104.2	29.7	39.9	23.0	73.8	99.2	25.4	34.4
25.8	73.1	105.3	32.2	44.0	23.0	73.8	99.3	25.5	34.6
25.5	72.9	105.9	33.0	45.3	22.5	72.7	97.1	24.4	33.6
25.8	73.5	105.6	32.1	43.7		72.9	101.8	28.9	39.7
25.0	74.8	105.9	31.1	41.6	25.6	75.9	106.6	30.7	40.5
24.8	73.6	108.1	34.5	46.8	26.0	71.4	99.7	28.3	39.6
25.0	71.9	101.1	29.2	40.6	25.5	70.2	98.7	28.5	40.6
24.7	72.0	102.4	30.4	42.2	23.0	73.1	106.2	33.1	45.3
	72.2	96.6	24.4	33.8	23.0	73.6	107.0	33.4	45.4
24.6	75.1	105.3	30.2	40.3	22.9	73.1	96.0	22.9	30.9
25.5	73.6	104.1	30.5	41.4	23.8	74.1	106.2	32.1	43.3
25.0	73.1	100.5	27.4	37.5	23.6	75.0	106.7	31.7	42.3
24.0	71.1	93.9	22.8	32.1	23.5	74.6	108.1	33.5	44.9
24.0	75.0	101.1	26.1	34.8	23.6	76.9	103.5	26.6	34.6
23.8	74.8	104.8	30.0	40.1	23.8	75.2	106.3	32.9	42.4
23.7	71.9	97.7	25.8	35.9	23.8	73.4	104.6	31.2	42.5
23.2	73.6	99.6	26.0	35.4	24.9	73.8	102.0	28.2	38.2
23.0	72.2	96.9	24.7	34.2		74.2	101.7	27.5	37.0
23.0	73.4	94.6	21.2	28.9	25.0	72.7	102.0	29.3	40.3

At R.T. of 22.5° C.–26.0° C., the mitotic elongation is 39.4 per cent; or, an egg 74.0 μ in diameter elongates to 103.2 μ.

yet the shape of these two curves is quite similar. Indeed, if one plots on the same graph all the time-elongation curves for eggs divested of their fertilization membranes, then one finds that the width of the area covered by their ascending slopes lies within the limits of variation prescribed by the values for the maximum elongation.

For convenience of analysis the curve, Fig. 2, may be divided into three parts. The first, the "incipient period of elongation," involves the slow extension that takes place during the elongation of the spindle (diaster), and the so-called "karyokinetische Streckung" (not neces-

sarily synchronous processes). The slight rise shown during this period was not found in every case studied, but its occurrence was so frequent and definite that it has been plotted as such, and not smoothed out. This "incipient" stage is the most interesting, and yet the most difficult stage to determine. It is hoped that future experimentation will furnish a more lucid analysis.

The second stage is marked by the rapid ascent of the curve and its completion coincides with the pulling-apart of the daughter-cells (they are actually always contiguous). Some time before reaching the peak the steeply-rising curve shows a decided inflection; from this point the curve approaches the maximum much more slowly. The

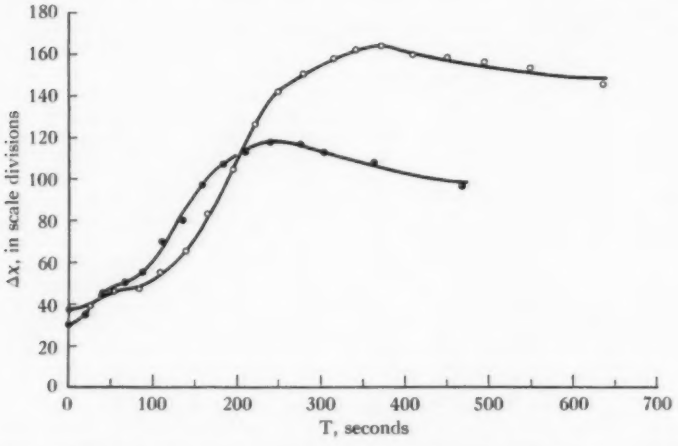

FIG. 2. Elongation of eggs after removal of fertilization membrane. Open circles, max. $\Delta x = 30.7\mu$; closed circles, max. $\Delta x = 22.9\mu$.

inflection indicates the time when the resisting influence of the hyaline-plasma membrane enters the picture. One may observe folds and wrinkles in this membrane at this period, and these probably indicate tension. Because of the fear of straying too far from the physiological picture, no attempt at removing this membrane was made—it can readily be removed by repeated washings in an isotonic (0.53M) potassium chloride solution.

The curves that have been plotted of normal eggs (with and without fertilization membranes) are to be used as a standard. Experimental work on the effect of various agents on the time-elongation curves are now in progress. It is hoped that this method of attack

will ultimately contribute to our knowledge of one phase of cell division; namely, the process of elongation.

The final feature of the curve is the "recovery," and is of no particular interest except to show that, at least for the length of time observed, the egg does not return to its original diameter.

Summary

For the experimental temperatures (22.2° C.–26.0° C.), curves have been plotted to show the course of mitotic elongation in the *Arbacia* egg at first cleavage. For eggs possessing fertilization membranes, the elongation is 11.5 per cent (i.e., an egg 74.0 μ in diameter elongates to 82.5 μ). When eggs are divested of their fertilization membranes, the elongation is 39.4 per cent—or, an egg 74.0 μ in diameter elongates to 103.2 μ. The data obtained are being used as a basis for further experimentation.

RESPONSE TO LIGHT IN PERANEMA TRICHOPHORUM

I. Relation between Dark-Adaptation and Sensitivity to Light

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(From the Zoölogical Laboratory of the Johns Hopkins University)

Introduction

It is well known that if photosensitive animals are subjected to darkness they become more sensitive to light, but there is much diversity of opinion concerning the relation between the time in darkness and the extent of change in sensitivity.

Hecht (1919, 1927) concludes that in *Mya arenaria*, *Pholas dactylus*, *Ciona intestinalis* and *Homo sapiens*, as the time in darkness increases, the sensitivity increases at first rapidly, then slowly, until it reaches a maximum, and then remains constant. Similar conclusions were reached by Obreshkove (1921) for *Rana clamitans*, Folger (1925) for *Amœba proteus*, Crozier and Wolf for *Agriolimax campestris*, and Hartline for *Limulus polyphemus*.

Dolley (1929) found in observations on *Eristalis tenax*, that as the time in darkness increases, the sensitivity increases to a maximum in about sixty minutes, then remains nearly constant for two hours, then decreases rapidly to a point lower than that which obtains for light-adapted specimens.

Mast (1932) maintains that in *Volvox* the relation between sensitivity and dark-adaptation depends upon the extent of preceding illumination. He says that if colonies have been in strong light one or two hours before they are subjected to darkness the sensitivity increases to maximum in approximately sixteen minutes and then decreases, but that if they have been in strong light nine hours the reverse obtains, i.e. it decreases to minimum in approximately sixteen minutes and then increases.

Material and Methods

The *Peranemæ* used in the observations described below were cultured in the laboratory. About 250 cc. pond water and 2 grams of beef suet were put into each of several finger bowls. The finger bowls were then covered and left one week or more after which a few *Peranemæ* were added to each. The *Peranemæ* in these cultures became very

abundant in a week and continued in good condition for a month or more.

The observations were made in a basement darkroom in which the temperature was 21 ± ½° C. A 1000-watt, 120-volt projection lamp was mounted in a ventilating shaft in front of an opening 2 cm. square. This lamp was in a circuit maintained at 110 volts, by means of variable

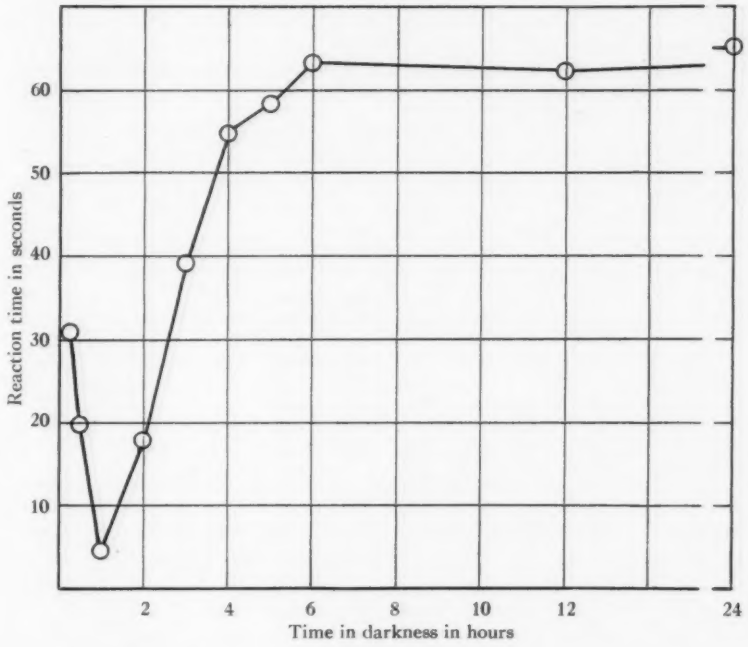

FIG. 1. Graph showing the effect of dark-adaptation on sensitivity to light in *Peranema trichophorum*. Each point on the curve, except the last two, represents the average reaction-time for 15 to 17 tests. The last two represent the average for 3 tests each (see Table I).

resistance. A small ruby lamp was mounted in front of the microscope a little above the beam of light from the 1000-watt lamp. The light from the two lamps was, by means of a front surface silvered mirror, reflected up through a compound microscope which had no substage condenser. The intensity of light from the 1000-watt lamp was, on the stage of the microscope, 2,000 meter candles. The red light from the ruby lamp was very weak.

A shutter and a 4 cm. glass heat screen, filled with water, were mounted in the beam of light near the shaft. The shutter contained a sliding panel with two opaque plates and an opening 3 cm. square between them. This panel was mounted in a frame in such a way that it could be readily raised and released by means of cords which extended to the microscope. It had a 50-gram weight attached to the bottom to increase the rate of fall.

TABLE I

Relation between dark-adaptation and sensitivity to light in *Peranema trichophorum*. Different individuals were used for each observation. The intensity of the light used was 2,000 meter candles.

Series	Time in darkness in hours									
	¼	½	1	2	3	4	5	6	12	24
	Reaction-time in seconds									
1	33.5	18.0	5.1	17.5	31.9	51.9	66.1			
	38.0	19.4	3.6	18.4	29.1	61.1	71.7	51.2		
	28.8	17.2	4.2	19.2	31.6	47.8	74.9	67.6		
	38.6	19.7	3.1	29.1	27.4	53.9	52.3	64.1		
2	29.1	23.2	2.4	11.4	42.4	44.2	54.6	77.2		
	31.7	17.6	4.1	17.6	37.1	49.8	46.8	68.2		
	26.4	14.1	4.8	21.9	29.2			59.4		
	27.2	22.8	3.9	15.4						
3	38.4	22.1	6.1	19.8	54.1	58.6	41.6	59.4		
	29.2	20.1	3.9	11.7	44.3	48.1	62.8	67.1		
	32.6	14.6	5.4	15.4		68.0	74.0	78.4		
4	32.1	19.7	4.8	22.7	59.6	49.2	51.6	54.3		
	26.4	25.2	3.1	15.9	39.7	58.3	67.4	72.3		
	30.2	17.4	5.1	18.1	41.2	71.1	62.1	64.1		
5	31.1	21.6	7.1	18.9	34.6	42.6	56.1	61.1	69.3	59.3
	26.2	17.4	3.9	23.4	32.1	59.7	47.0	60.4	57.8	72.8
	29.1	24.1	5.4	17.1	34.4	68.2	64.3	48.3	60.6	64.3
Total avg.	30.95	19.8	4.54	17.94	39.19	55.09	58.58	63.46	62.56	65.47

The observations were made as follows: Some fluid was taken from a flourishing *Peranema* culture and filtered; *Peranemæ* were then taken from this culture and one was mounted on each of 24 to 30 slides, in a drop of the filtered fluid under a cover-glass supported with a vaseline ring. These slides were now taken to the darkroom and put into a light-tight moist chamber. After several minutes the 1000-

watt and the ruby lamps were turned on, the panel raised (so as to intercept the light from the 1000-watt lamp), one of the slides transferred to the stage of the microscope and the *Peranema* brought to focus in the red light. The *Peranema* was then suddenly exposed to light from the 1000-watt lamp by releasing the panel and the time required to induce a response measured. This was repeated with each of the other *Peranemæ* on the slides after having been in darkness different periods of time. These specimens were then discarded. This whole process was repeated four times. In four of the five series of observations the time in darkness extended to six hours and in one to twenty-four hours.

The response in *Peranema* to sudden increase in luminous intensity is marked and precise. It consists of sharp deflection of the anterior end of an "all-or-none" type. This made it possible to measure the reaction-time fairly accurately.

The cultures were kept in a north window. The experiments were made in April and May and each one was started about 2 o'clock in the afternoon. The *Peranemæ* used in all the experiments were therefore thoroughly adapted to fairly strong diffuse daylight when they were taken to the darkroom.

Results

The results obtained are presented in Table I and Fig. 1.

Table I shows that there was remarkably little variation in the reaction observed in the different individuals used. The probable error was only 0.18 to 3.88. This table and Fig. 1 show that as the time in darkness increased, the reaction-time decreased to a minimum in about one hour, then increased to maximum in about three hours and then remained nearly constant for at least twenty hours.

Summary

If light-adapted specimens of *Peranema trichophorum* are subjected to darkness, the sensitivity to light increases rapidly to maximum (as the time in darkness increases), then decreases slowly to minimum after which it remains nearly constant. The sensitivity reaches maximum after about one hour in darkness and minimum after about four hours.

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PULSATION OF BLOOD VESSELS IN OYSTERS, OSTREA LURIDA AND O. GIGAS[1]

A. E. HOPKINS

U. S. BUREAU OF FISHERIES

Blood vessels are recognized to undergo frequent changes in diameter as a result of activity of muscle fibres in their walls. The capillaries of vertebrates, although of relatively simple structure, are highly variable, due to the reactions of "Rouget cells" along their walls (Krogh, 1922). Similar cells were described by Parker (1923) in an annelid, *Nereis*. In this species Federighi (1927, 1928) described the structure of the pulsating dorsal blood vessel and "hearts" as being activated by cells ranging from simple stellate form to typical smooth muscle cells.

In lamellibranchs, however, few blood vessels are found which have the definite and characteristic walls possessed by annelids as well as vertebrates. Most of the circulatory system consists of relatively indefinite spaces within the tissues. Even an endothelium appears to be lacking save in such vessels as those in the gill filaments (Kellogg, 1890).

Description was recently published (Hopkins 1934*a*, 1934*b*) of a pair of large peripheral vessels, or "accessory hearts," in the walls of the mantle which pulsate rhythmically. This finding is of interest in view of the general absence of definite walls in the peripheral vessels. The present study was made for the purpose of throwing some light on both the structure of pulsating vessels and their mode of activity.

At the time of writing this paper a publication (Elsey, 1935) has just appeared which interprets the activity of the "accessory hearts" as pumping blood from the gills and circumpallial arteries into the auricles. Elsey stated (p. 149), "Although it has not been possible to trace the direction of the blood in the pulsating organs, the fact that they are, through their connection with the pallial veins, in communication with the auricles, leaves scarcely any doubt that part of the blood from the gills and circumpallial blood vessels passes through them toward the heart and that subsequently this blood with that of the main pallial vein is forced by contractions of the accessory pulsating organs into the auricles."

[1] Published by permission of the Commissioner of Fisheries.

Pulsating Vessels in Ostrea lurida

Observation of the activity of blood vessels in the mantle of the intact oyster is difficult because of the heavily calcified shell. The accessory hearts were made visible by sawing away portions of one valve, allowing the mantle to curl back over the cut edge. However, this method did not permit microscopic examination by transmitted light whereby it would be possible to observe the movement of blood corpuscles.

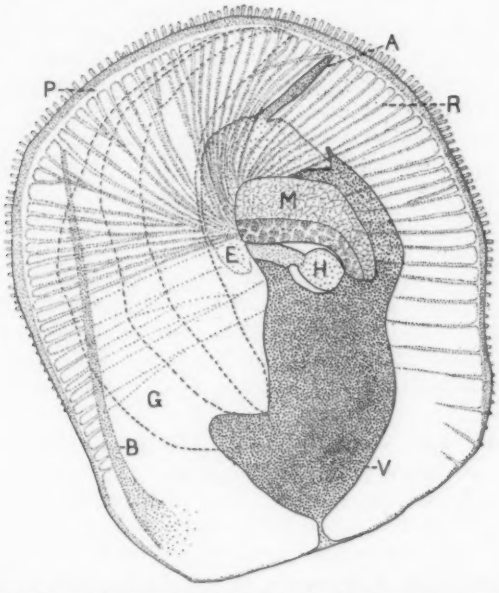

Fig. 1. Right mantle of oyster about 1 cm. in diameter as seen by transmitted light. *A*, accessory heart; *B*, waste canal; *E*, excretory organ; *G*, outline of underlying gills; *H*, heart; *M*, adductor muscle; *P*, circumpallial sinus; *R*, radial vessels; *V*, visceral mass.

In order to make such a study, glass plates were placed upon oyster grounds near Olympia, Washington, and the oysters which caught on them were allowed to grow to diameters ranging up to about two centimeters. A plate could be placed directly upon the stage of the microscope, the upper (right) valve of the specimen scraped clean, and the tissues observed uninjured by means of a bright transmitted light. Oysters about one centimeter in diameter were most satisfactory, for at this size the valves are not sufficiently calcified to render them opaque.

At the beginning of the study of such material it was a great surprise to the observer to see not only the actively pulsating heart and accessory hearts, but also a great number of smaller vessels pulsating with a slow but pronounced rhythmicity. One gets the impression that the entire mantle is involved, so numerous are the vessels and so distinct the waves of pulsation along them. Elsey (1935, p. 148) mentioned that, "Observation of young oysters from 1 to 2 millimeters in diameter showed that many of the radial blood vessels at this early stage pulsate in a manner quite similar to the accessory pulsating organs of the adult."

A drawing (Fig. 1) is reproduced to show the vessels of the right side of a specimen about one centimeter in diameter. At this size the general anatomy is that of the adult oyster. The heart lies just anterior to the adductor muscle. In the wall of the mantle the excretory organ (organ of Bojanus) adjoins the posterior and ventral borders of the muscle and extends forward under the pericardium. The accessory heart proceeds dorsally from the posterior portion of this organ. Radiating from the region of the adductor muscle and excretory organ, and anteriorly from the visceral mass, are a great many well-defined bands (*R*). Each one of these consists of a bundle of muscle fibres, the retractor muscles of the mantle. Each bundle of fibres appears to be associated with and partially surrounded by a blood vessel which pulsates rhythmically (Fig. 2).

Another active vessel is associated with the waste canal (*B*), which runs as a prominent band from near the labial palps at the anterior end to a point near the ventro-posterior edge of the mantle, where it becomes continuous with one of the radial vessels. The epithelium of the waste canal is ciliated, the cilia beating posteriorly while those of the adjoining mantle surface beat toward this band. The waste canal is both structurally and functionally identical to similar organs found particularly in lamellibranchs with well-developed siphons (Kellogg, 1915), though in the case of *Ostrea lurida* the edges are not as prominently erect. In *O. gigas*, the structure seems to have disappeared entirely. Associated with the waste canal throughout most of its length, except near the anterior end, is a blood vessel which may be observed to pulsate.

All of these vessels, including the accessory hearts, open peripherally into the vessel which runs around the margin of the mantle lobe. In some species such as *O. angulata* (Leenhardt, 1926) and *O. gigas* (Hopkins, 1934) this vessel may correctly be called the pallial or circumpallial artery, for the heart pumps blood directly into it. In *O. lurida*, however, it would appear more correct to call it the circum-

pallial sinus. Although a great many attempts were made to inject a colored solution into this vessel by way of the ventricle, as is readily possible with *O. gigas*, no success was had. Also, branches of the aorta spread fan-like into the mantle tissues of *O. lurida* though not of *O. gigas*. In the latter species the circumpallial vessel is very large and prominent anteriorly and relatively small along the posterior border. In fact, even in the shells of large specimens the imprint of this structure may be seen as a shallow groove. In the former species

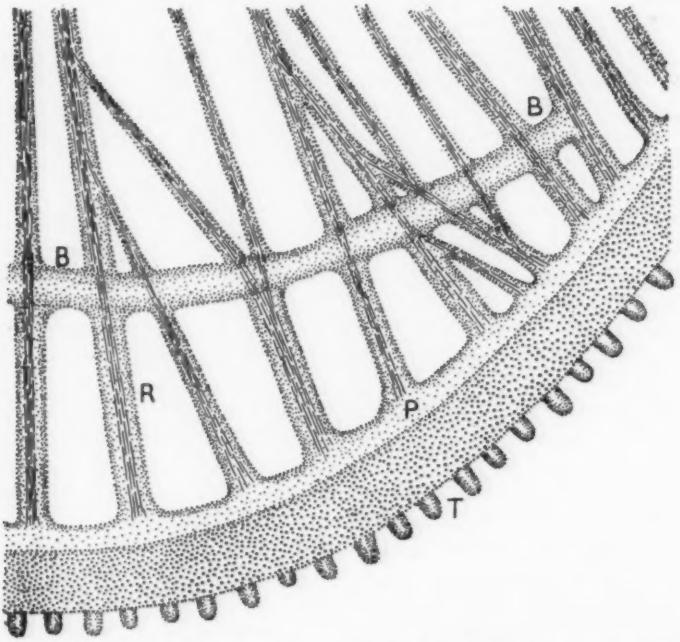

FIG. 2. Drawing of portion of inner surface of left mantle showing radial bands of muscle fibres associated with radial vessels (*R*). *B*, posterior end of waste canal; *P*, circumpallial sinus; *T*, tentacles.

the vessel is prominent posteriorly and seems almost to disappear at the anterior end.

Whether the circumpallial sinus also pulsates independently is not clear, though its diameter changes tremendously as waves of pulsation pass over the radial vessels. It appears probable that this vessel also is active since it is similar in structure to the others as well as continuous with them.

In a previous work (Hopkins, 1934) in which the accessory hearts were described, it was stated that the wave of pulsation originates at the central end, where the accessory heart joins the kidney, and travels slowly distally. At that time the observer was unable to watch the actual movement of blood corpuscles. The small specimens of *O. lurida*, attached to glass, gave opportunity to determine the direction of flow of blood during pulsation.

As indicated in Fig. 1, the accessory heart is directly continuous with the tissues of the excretory organ. The latter consists of a sinus in which the nephridial tubules are suspended. Blood corpuscles may be seen moving about in the blood spaces of the excretory organ and into the accessory heart. A wave of contraction starts apparently within the excretory organ itself and extends outward along the accessory heart forcing the blood rapidly ahead of it. As relaxation occurs corpuscles may be seen entering the accessory heart from both distal and proximal ends, indicating the absence of an effective system of valves. In some cases relaxation involves a definite flow of blood back into the excretory organ, though such does not seem to be typical. This reversed direction of flow is probably traceable to pressure in the circumpallial sinus created by discharge into it of other vessels.

The activity of the radial vessels is essentially similar to that of the accessory hearts in that the wave of constriction and the most rapid flow of blood originate centrally and travel to the periphery of the mantle. Absence of valves permits considerable return of blood on relaxation.

Because of the difficulty of seeing fine details, the origin of blood entering the radial vessels is still not clear. At least some of those in the posterior region definitely receive blood from the excretory organ, as in the case of the accessory heart. Prominent corpuscles have frequently been observed to enter the excretory organ on relaxation of the accessory heart and proceed distally through a neighboring radial vessel. However, the tissues of the whole region of the mantle in the vicinity of the adductor muscle and excretory organ consist of a network of irregular blood spaces which are continuous with the radial vessels and probably also with the excretory organ.

While blood in these pulsating vessels may be observed to move back and forth, according to the phase of pulsation, non-pulsating pallial veins may be observed in the same region which are returning blood from the mantle directly to the auricle. Blood in these veins moves only in one direction, in a periodic manner due to activity of the heart. These veins are clearly defined and entirely different from

the pulsating vessels, and they are adjacent to the outer surface of the mantle next to the shell, while the others are near the inner surface.

Activity of the longitudinal vessel which underlies the waste canal (Figs. 1 and 2, *B*) begins anteriorly and travels as a wave of pulsation which appears to be coördinated with pulsation of radial vessels crossing it and which in at least some cases are continuous with it. Blood corpuscles have been observed to flow from the waste canal vessel into radial vessels, though there is some flow in the reverse direction during relaxation. Anteriorly many of the radial vessels appear to originate in the waste canal vessel, though it is possible that they may be in connection with more central blood spaces which are not prominent enough to be seen.

The activity of all of the pulsating vessels appears to be loosely coördinated. They do not all pulsate synchronously, but rather as if a progressive wave slowly involves them. It has not been possible to trace the origin of a wave of transmission from vessel to vessel, and it is unknown whether the pulsation originates centrally or within the vessels themselves. Its very slow rate is suggestive of the activity of smooth muscle. The rate of pulsation is approximately the same as that of the accessory heart, and probably identical with it since the latter appears to be merely the most prominent of such vessels.

The circumpallial sinus is observed with some difficulty because of the pigmentation of this portion of the mantle. Whether it actually pulsates is therefore questionable. Blood may be observed to flow for a few seconds in one direction and then reverse itself, depending upon the phase of pulsation of the radial vessels. The pulse to be observed is that of the radial vessels and accessory heart and is much slower than that of the systemic heart.

The observations described above were made primarily upon small oysters, or "spat." However, these active vessels are not confined to the small specimens. They have been observed in adult oysters as well, though in this case the movements of blood corpuscles were not readily observable. By removing the right valve of an adult specimen and allowing the right mantle lobe to curl up out of the way, it was possible to observe the activity of vessels in the left lobe of the mantle.

The radial vessels are clearly associated with the radial retractor muscle bundles of the mantle. In Fig. 2 is a drawing of a portion of the mantle showing the posterior end of the waste canal and the radial muscle bundles and vessels. It will be noted that the muscle bundles are covered with and partly surrounded by the blood vessels. These structures stand up as pronounced ridges on the inner mantle surface, particularly when the vessels are in the expanded

condition. The vessels are larger distally than centrally and receive numerous small vessels, not shown in the figure.

Independent Activity of Accessory Hearts

The question arose as to whether the rhythmicity of such peripheral vessels as accessory hearts originates centrally or within the organs themselves. It was found in a previous work (Hopkins, 1934) that the rate of pulsation of the accessory hearts in *O. gigas* is roughly about one-third that of the heart. It is reasonable to assume that the rate of activity of such distal vessels would be dependent upon the rate at which they fill with blood, since stretching smooth muscle characteristically stimulates contraction. However, it would be interesting to determine if pulsation occurs at all when the organs are separated from the systemic circulation.

For this purpose use was made of large Japanese oysters (*O. gigas*), in which the accessory hearts are 30 to 40 millimeters long. A specimen was removed from its shell, and a portion of the mantle wall of the cloacal chamber, including an accessory heart and a portion of the distal end of the gills, was excised. This was kept in running sea water. When the mantle was cut all of the blood vessels as well as the retractor muscles became contracted. The accessory heart, which had a diameter when expanded of 3 or 4 millimeters, contracted to about one millimeter in thickness.

The specimen remained in the contracted state for from 36 to 48 hours before the accessory heart showed any indications of relaxation. However, as soon as this organ began to expand one could see feeble pulsating movements occasionally at the distal, uninjured end. Thereafter it progressively expanded, within about a day, to what appeared to be the normal diameter, and rhythmic pulsation became more pronounced and finally included the entire vessel.

Excised portions of mantle like that just described were prepared in several instances. They lived well as long as they were kept in sediment-free, running sea water. They have been kept in good condition for periods as long as 30 days. Death in all cases occurred as a result either of stoppage of the water supply or the presence of a quantity of sediment which permitted bacteria to get a foothold. The specimens were of interest in that they exhibited apparently normal activity. The tentacles frequently moved and were sensitive to stimulation by chemicals and touch, and even reacted to a decrease in the intensity of light. The cilia of both mantle and gills were active and the latter pumped water. Due to the action of cilia, specimens moved about all over the dish in which they were kept. On the outer surface of the mantle secretion of shell was well begun.

The accessory heart pulsated with a slow rhythm and at a rate comparable to that of such organs in the intact oyster (Hopkins, 1934).

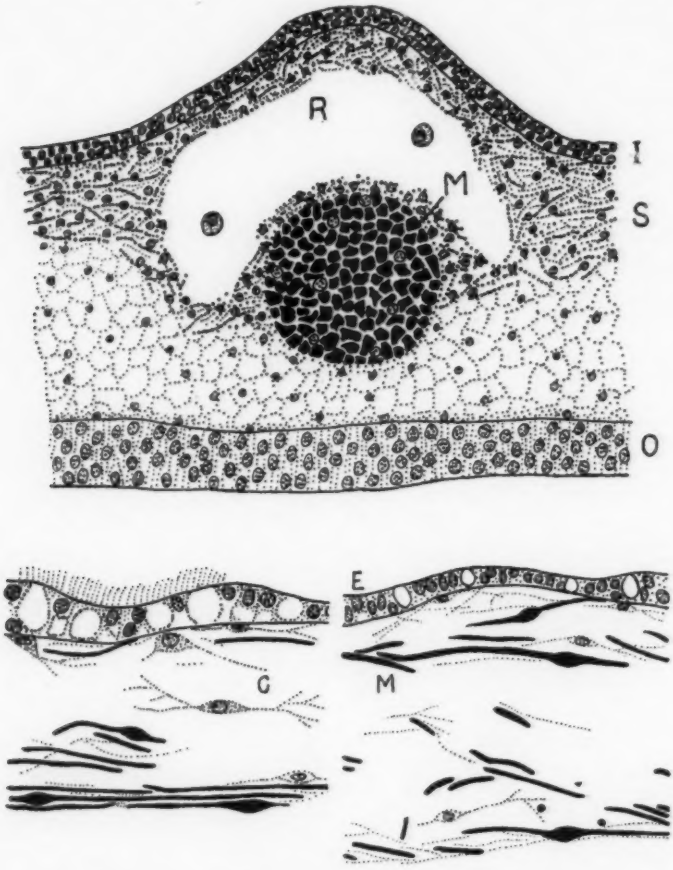

FIG. 3. Semi-diagrammatic drawing of portion of cross-section of mantle of *Ostrea lurida*, showing radial vessel (*R*) associated with radial band of muscle fibres (*M*). *I*, epithelium of inner, and *O*, of outer surfaces of mantle; *S*, sub-epithelial matrix of connective tissue and smooth muscle fibres.

FIG. 4. Two portions of wall of expanded accessory heart of *O. gigas*. *E*, epithelium; *C*, connective tissue cell; *M*, smooth muscle cells.

After the excised portion had become well adapted, the wave of pulsation was obviously from the central to the distal end. The rate

of transmission of the wave as measured over a length of about 30 millimeters was generally from 4 to 8 millimeters per second at 12° to 15° C., though actual contraction was not completed for 3 to 4 seconds more. Such a slow rate of conduction compares favorably with the rate of 5 to 20 millimeters per second given by Clark (1927) for conduction by the plain muscle of the frog's stomach at 15° C.

Structure of Pulsating Vessels of Mantle

The high degree of active pulsation exhibited by the numerous blood vessels in the walls of the mantle is surprising in view of the indefinite nature of most peripheral blood channels in lamellibranchs. It is generally known that the main arteries break up into blood spaces within the tissues. After observing the activity of the mantle vessels, it was thought likely that microscopic study would show them to be definitely walled, regular vessels, similar to the "hearts" of *Nereis*, with an endothelium and circular muscle fibres.

Serial sections were made of entire Olympia oysters (*O. lurida*) and of one of the isolated portions of mantle described above. Sections were stained either with Ehrlich's hæmatoxylin or Heidenhain's iron hæmatoxylin, with eosin as counterstain.

The epithelium of the inner mantle surface is simple, consisting of a single layer of cells which vary from very thin to semi-columnar. Goblet-cell mucus glands are abundant and some of the cells, chiefly the longer type, are ciliated. Underlying the epithelium is a matrix of connective tissue in which numerous smooth muscle cells may be seen (Fig. 3).

The sub-epithelial tissue appears as a loose network in which open spaces of all sizes and shapes with indefinite boundaries occur. These are the blood spaces or "vessels." Normally fixation of a specimen results in the contraction of many of these spaces, in which case one can see no histological indication of what was, in the expanded condition, possibly a prominent radial vessel. Most of the sections across the radial vessels (Figs. 2 and 3) show well the bundles of radial muscle fibres, but only occasionally is the cavity of the blood space thoroughly open. Kellogg (1890) figured the accessory hearts in cross-section but described them as ridges, probably because the contraction resulting from fixation left no remaining cavity. Yet at least the larger of the vessels are obviously definite in location. The spaces may not be seen when in a state of contraction only because of the absence of well-formed walls.

Sections of the accessory heart (Fig. 4) of one of the isolated pieces of mantle, fixed in a state of complete expansion, show more clearly

the location and mode of functioning of the sub-epithelial smooth muscle fibres. The cavity of the vessel appears to be in the middle of the network of tissue so that when expanded the outer wall is suggestive of a well-defined structure. The muscle fibres appear to run in all directions. In certain cases it has been thought that an endothelium was present, but this was probably only the connective tissue fibres stretched by expansion of the vessel. Frequently one may see either muscle fibres or strands of connective tissue extending across the cavity of an expanded vessel. Elsey (1935) considers the sub-epithelial muscle fibres of the accessory hearts to constitute circular muscle. This may be correct, but it is possible that such an appearance may be due only to the expansion of the vessels and consequent stretching of fibres.

Discussion

Activity of blood vessels is generally associated with a definitely walled structure of a specific nature, as in the typical arteries, veins, and capillaries. Lacking such structure and consisting only of irregular spaces within the tissues, the blood channels of the oyster are subject to changes in diameter of a rhythmic nature which result in producing a flow of blood within the tissues of the mantle. It would seem hardly probable that such pronounced rhythmic activity of peripheral vessels as that described above is a peculiarity of oysters alone, or even of lamellibranchs.

The inner surfaces of the mantle of lamellibranchs, with their rich supply of blood spaces, are commonly considered to constitute the principle organ of respiration, while the gills function largely in the feeding activity and have a relatively slight blood supply. On observing under proper conditions the flow of blood through the spaces in the tissues of the mantle of *Ostrea lurida* as influenced by rhythmic contractions of the relatively unformed and indefinite vessels, one readily agrees that the mantle may constitute the primary respiratory organ. Although it would be difficult to observe, it is considered probable that blood spaces throughout the body of the oyster are similarly active, for smooth muscle fibres may frequently be seen near them.

It was suggested (Hopkins, 1934) that the accessory hearts of the oyster may be homologous to the "gill hearts" of cephalopods, and it may be that this is the correct interpretation from the evolutionary point of view. One may think of a particular pair of the active radial vessels becoming larger as they take over more and more of the function of supplying blood to the organs of respiration. In *O. gigas*

the radial vessels, with the exception of the accessory hearts, are less prominent or, at least less readily observed to pulsate, than in *O. lurida*. In the former species the heart pumps blood directly into the pallial artery into which the accessory hearts also empty, thereby possibly functioning to supply the tissues of the mantle with the blood required for respiration. This blood supply is provided in the latter species primarily by the pulsating radial vessels.

Elsey's (1935) interpretation of the function of the accessory hearts in *O. gigas* is that they pump blood into the auricles, rather than from the blood spaces of the excretory organs into the circumpallial artery, as previously described (Hopkins, 1934). He gave no evidence in support of the hypothesis save that proximally they communicate with pallial veins. Although it must be admitted that the function of these organs is not yet entirely clear, it is difficult to understand why such large, active structures should be developed in an organism to pump blood from a large artery (the circumpallial) directly back to the auricles. However, he did not describe either the direction of the impulse of contraction or the blood corpuscles in motion.

The mechanism whereby the gills of most lamellibranchs receive an adequate blood supply is generally not clear. Grave (1909) described the mode of circulation through the gill lamellæ of *Atrina* in detail. Blood enters the reflexed lamellæ from a large vein originating in the kidney and leaves through the direct lamellæ. Since the blood from the ventricle passes first through the tissues of the viscera before being collected in the spaces of the kidney, it is probable that a pulsation of the afferent veins of the gills would be necessary to force the blood through the small vessels of the gill filaments.

Similarly, in *Mytilus* the arrangement of afferent and efferent veins of the gills would appear to imply the feeble circulation which Field (1922) ascribes to them unless the afferent vessels are capable of pulsation. Kellogg (1890) observed the blood of *Arca* to flow back and forth through individual gill filaments. Corpuscles were observed to move outward for about half a minute and then reverse their direction.

These examples are selected from the extensive literature on the subject merely to suggest that an understanding of the possible pulsating activity of afferent branchial veins might result in clearer ideas of the physiology of lamellibranch gills. Although the accessory hearts of the oyster may pump some blood into the gills (Hopkins, 1934), it appears that these organs are not the chief source of the branchial circulation, as pointed out by Elsey (1935). The blood

vessels of the gills are directly continuous with the large veins to which they are attached and which return blood from the visceral mass to the excretory organs. These large veins are difficult to observe in the intact oyster, but they have frequently been seen to change their diameter slowly, at times with what appeared to be a sluggish rhythmicity, suggesting that a back and forth movement of blood through the filaments might result. Observations by Elsey (1935) indicate that blood vessels in the principle filaments of the gills react to stimulation with a consequent reduction in diameter.

The difficulty in detecting pulsation of peripheral blood vessels may well be due to their very slow rate. A pulsating activity with a periodicity of several minutes would not be identified as rhythmic, though it might be as much so as the activity of the heart itself. In several instances series of stop-watch readings have been taken of the slow expansion and contraction of the large medial vein to which the inner gills are attached. Although the exact time of beginning and ending of any phase could not be determined, the measurements for a complete contraction at 15° C. vary between 100 and 150 seconds. Whether this activity constitutes rhythmicity is not established, though it appears probable.

Summary

1. In the mantle of *Ostrea lurida* is a series of slowly pulsating blood vessels.

2. The many radial vessels are associated with the bundles of retractor muscle fibres of the mantle. The "accessory hearts" appear to be similar to these, though much larger.

3. The waste canal, a band of tissue running anteroposteriorly in the ventral wall of each mantle lobe and covered with cilia beating posteriorly, overlies a pulsating vessel which crosses and is continuous with many radial vessels.

4. All of the pulsating vessels communicate distally with the circumpallial sinus, which appears in this species not to be directly connected with the heart.

5. The vessels described pulsate at a slow rate and their activity is loosely coördinated.

6. The wave of contraction of the radial vessels originates centrally and travels slowly distally, forcing the blood outward into the circumpallial sinus. The pulsation of the vessel underlying the waste canal appears to begin at the anterior end.

7. The absence of effective valves is shown by the reversal of direction of flow of the blood as relaxation takes place.

8. The blood supply of the radial vessels appears to come from the blood spaces of the excretory organs and nearby tissues.

9. Isolated portions of the mantle of *O. gigas*, including an accessory heart, live for weeks, the accessory heart pulsating at approximately its normal rate. The rate of transmission of the impulse of contraction is slow, 4 to 8 mm. per second. The activity is compared to that of vertebrate smooth muscle.

10. The pulsating vessels appear to be indefinite spaces within the sub-epithelial matrix of connective tissue and smooth muscle, and an endothelium and other well-defined structures characteristic of formed blood vessels may be lacking.

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THE PRECISE CONTROL OF GROWTH IN A PURE CULTURE OF A CILIATE, GLAUCOMA PYRIFORMIS[1]

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INTRODUCTION

Biologists have long demanded cultures of animal cells for a wide variety of investigations. In the past the usefulness of protozoans for biological research has been greatly limited by the necessity of bacteria for food. None of the larger forms has been grown in the absence of other life, i.e., pure culture. However, a small number which can be handled readily as individuals when magnified only a few diameters under the dissecting binocular microscope has been recently obtained in pure culture, and some of these have already proved useful.

The present paper describes a medium which supports extremely vigorous growth of *Glaucoma* in pure culture. It presents a quantitative analysis of the characteristics of growth of *Glaucoma*, and shows that the control engendered by the culture method permits duplication of similar experiments as frequently as desired.

IDENTIFICATION

The holotrichous ciliate *Glaucoma pyriformis* is a facultative parasite and is capable of growth under strictly anærobic conditions.

Figure 1 contrasts *Glaucoma* (*a*) with *Colpidium campylum* (*b*), the only ciliate which, in my experience, can cause confusion. *Glaucoma pyriformis* is distinguished by a conspicuous oral membrane, negligible gullet, and a round nucleus. In addition it grows vigorously in Difco yeast extract, while *Colpidium campylum* has never been grown in pure culture.[2]

Dr. André Lwoff very kindly compared my animal with his

[1] A grant of money from the Brooks Fund defrayed a portion of the cost of this work.

[2] The "*Colpidium campylum*" reported upon by the author in 1932 and 1933, used at Stanford, and carried to Yale by Dr. Bond, is a typical *Glaucoma pyriformis*. A sub-culture of this strain was used by Elliott in his 1935 paper (personal communications from Dr. Jahn and Dr. Bond). Chatton never achieved the pure culture of *Colpidium campylum* (personal communication). The author has tried to grow this ciliate on the media described in the present paper without success—its nutritive requirements resemble those of *Colpidium colpoda*.

Glaucoma pyriformis, using his precise silver impregnation method. He found extremely slight differences in the silverlines. He also compared Dr. Butterfield's (1929) "*Colpidium*" with my *Glaucoma pyriformis*, finding no appreciable difference.

Dr. A. Kahl also advised me concerning the identification of this ciliate. He identifies it with his *Saprophilus oviformis* (Kahl, 1926, 1931), apparently synonymous with *Glaucoma pyriformis*. The species *Colpidium striatum*, inadequately described by Stokes (1886), he treats as a nom. nud.

Unfortunately the taxonomy of this species is not settled as yet, so the most well-known name is used here.

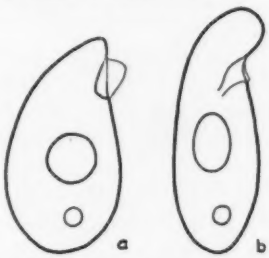

FIG. 1. *a*, *Glaucoma pyriformis* (Ehrb.) Schew.: 47μ. *b*, *Colpidium campylum* (Stokes) Bresslau: 55μ.

MATERIALS AND METHOD

General

The temperature for all experiments varied between 24.2° and 25.2° C.

Bacteriological methods for sterilization of apparatus and manipulation of organisms (flame technique) were used. A method for sterilizing protozoans has been reported (Hetherington, 1934*a*).

Glassware

Stock and experimental vessels were 125 cc. Pyrex Erlenmeyer flasks. Isolation vessels were Columbia dishes.[3] Graduated serological pipettes of 2-cc. capacity were used for stock transfer and for measuring inocula in the experiments.

Enrichment

Glaucoma pyriformis adjusts readily to 0.5 per cent yeast extract and proceeds to grow, while the other ciliates which have been obtained in pure culture are difficult to start in this medium.

[3] Arthur H. Thomas Co., Philadelphia, Watch Glass No. 9851.

Transfer

Daily transfer of approximately 0.2 cc. was continued for one month or more, thus maintaining fairly constant and extremely vigorous growth, previous to quantitative experimentation.

It is essential to observe the following procedure in measuring the volume of inocula. Draw up the 2-cc. volume and allow to flow back into the flask at the same rate at which the volumes will be delivered, then (keep the culture stirred!) again draw up, slowly (i.e. at the speed at which it will be discharged), and deliver the desired volumes into the flasks. Do not use the final 0.4 cc. in the pipette.

Counting

A method of counting was described (Hetherington, 1934*b*). This has been refined to overcome a specific difficulty; a tendency on the part of *Glaucoma* to adhere to the surface of the pipette.

1. If dilution is required (if not, proceed directly with Step No. 2), draw up 10 cc. into a clean graduated pipette, then deliver 10 cc. back into the culture. Stir thoroughly. Again draw up 10 cc. and deliver into that volume of tap water in a beaker which will achieve the correct dilution.

2. Prepare 10 slides (or any number desired) by placing two separate drops of particle-free aceto-carmine on each.

3. Using a pipette (medicine dropper) whose drop-volume has been ascertained, draw up a volume larger than required for counting, and deliver drop by drop back into the culture. A convenient volume for each drop is 0.02 cc.

4. Stir thoroughly. Draw up a similar volume. Deliver one drop on top of each aceto-carmine drop, being careful to hold the pipette at the same angle at which it was calibrated. Do not deliver the final 2 or 3 drops.

The organisms were counted under the low power of a dissecting binocular. The number of organisms in the original culture can be calculated by obvious methods.

Accuracy of Counting Method

The following numbers are six successive counts, giving glaucomas per cc., from the same culture: 7,770; 7,070; 7,130; 7,710; 7,410; 7,610. This series shows the largest variation experienced. The mean is 7,450, with a probable error of 174.5, or 2.3 per cent. The majority of counts were more satisfactory than the above, giving a mean probable error of about 1 per cent.

The use of pipettes smaller than 2 cc. and with narrow delivery apertures decreases the accuracy because the ciliates adhere more irregularly.

Division Rate

The division rate, r, the number of divisions per organism per day, is given by the following formula:

$$r = \frac{\log Y - \log P}{t \cdot \log 2}$$

where Y = total yield of ciliates, P = inoculum, the number of ciliates inoculated, t = time of growth in days.

If young organisms or those about to divide (i.e. considered as an inoculum of 2 for each dividing individual) are inoculated the following formula must be applied:

$$r = \frac{\log Y - \log (0.7071P)}{t \cdot \log 2} .$$

The factor 0.7071 expresses the approximate difference between the age of the individuals of the inoculum and the mean age of those of the yield.

Medium

Inorganic

While this ciliate will, of course, grow on the complex nutrients, e.g. yeast extract dissolved in doubly distilled water, all experiments were carried out using a salt solution, Peters' medium, in place of the pure water. This medium, in addition to the very desirable buffering action, possesses at least the empiric virtue of resembling ordinary fresh waters, particularly with respect to its dilute nature and content of calcium bicarbonate.

Preparation

Put about ten grams of $Ca(OH)_2$ (Kahlbaum—*pro analysi*) into about a liter of doubly distilled water: cork and shake. Allow to stand for two days, shaking a few times in the interim. Pass CO_2 through this solution (at 23° C.), using either commercial tank CO_2 or a generator, for one hour. Cork and allow to stand for 24 hours. Pour off the slightly cloudy supernatant fluid into two good quality ground-glass-stoppered bottles. Pass CO_2 through each for a minute, stopper, and label: $Ca(HCO_3)_2$, 0.01 M., 55 cc./liter. Prepare 0.015 M. $MgSO_4$, K_2HPO_4, and 0.0075 M. Na_2HPO_4 in doubly distilled water, and add "10 cc./liter" to the label of each.

A liter of Peters' medium is prepared by diluting 55 cc. of the calcium bicarbonate and 10 cc. of each of the others to one liter. This medium should not be shaken into equilibrium with air before autoclaving.

Organic

The most striking characteristic of this particular strain of *Glaucoma pyriformis* is its complete failure to grow on peptone (or Neopeptone or Tryptone, Difco). Since it was found to grow luxuriantly on plain autoclaved wheat or on yeast (Fleischmann, pure dry), and since these media contain great numbers of particles, it was considered a "particle feeding" strain, and extensive measurements of growth were made using a medium of 0.5 per cent yeast, and 0.5 per cent dextrose.[4]

Since the advent of the new powdered yeast extract by Difco, it has been possible to show that particles are not the crucial requirement of my strain. Further, it has permitted a far closer approach to an ideal medium, particularly so when combined with certain powdered yeasts which were specially prepared under the direction of Mr. H. G. Dunham of the Difco Laboratories. Without his gracious coöperation in supplying these yeasts, the high division rates described in this paper could not have been achieved.

Clear 0.5 per cent Yeast Extract will support this *Glaucoma* with a division rate of 6.36. As stated, peptone will not support growth. What is the effect of adding a fine suspension of insoluble yeast particles to 0.5 per cent peptone? Difco extracted yeast no. B13412H was prepared for this purpose. When added in 0.005 to 0.05 per cent concentration, yielding cloudy suspensions, growth remained nil, just as in clear peptone. When raised to 0.1 per cent, however, considerable growth could be obtained. This is in contrast to growth in 0.5 per cent peptone + finely powdered whole yeast (No. B13412), where 0.005 to 0.05 per cent concentrations gave good growth. Raising the concentration to 0.1 per cent improved growth to a relatively small extent.

From these experiments it was concluded that minute quantities of the extractives of yeast complete peptone as a food for this strain of *Glaucoma pyriformis*, and that particles are of slight or no importance.

Peptone and yeast extract gave similar growth in the presence of 0.1 to 0.3 per cent whole yeast No. B13412. When the whole yeast

[4] Note the similarity of this experience with that of Peters (1929). It seems to the author practically certain that Peters' "*Colpidium colpoda*" is *Glaucoma pyriformis*.

was reduced to 0.05 per cent, the peptone medium gave weaker growth. The mean division rate on the 0.2 per cent and 0.1 per cent whole yeast + 0.5 per cent yeast extract was respectively 7.83 and 7.71. The following optimum medium was used for the measurements to be reported in this paper: 0.5 per cent yeast extract and 0.2 per cent powdered yeast No. B13412 in volumes of 50 cc. in 125-cc. Pyrex Erlenmeyer flasks. The surface/volume ratio is 0.55.

The finely powdered yeast is prepared in 1 per cent concentration which is autoclaved, 15 lbs. for 15 minutes. All particles must be wet before autoclaving, otherwise sterility may not be achieved. This is allowed to stand for 24 hours, then the cloudy supernatant fluid is poured off, separating from the solid residues. It is regulated to pH 7.0 to 7.2 with NaOH, and added to 1 per cent yeast extract in the correct proportion, then diluted: dispense into the flasks, cotton stopper, and autoclave.

Comparison of Different Media

Table I verifies quantitatively some of the relations just described, and permits comparison of a number of different nutrient media. Inoculum 15 is 1,490 glaucomas from a culture in 0.5 per cent yeast extract. Inoculum 7 is 717 from a culture in dextrose [5] 0.5 per cent, yeast No. B33425 0.25 per cent, and yeast No. B33413 0.25 per cent. The numbered yeasts are whole yeasts prepared in different ways. Cultures containing 0.5 per cent of whole yeast may for convenience be called whole yeast media, while those containing yeast extract may be called yeast extract media.

The most general relations observed are that inocula from whole yeast into whole yeast give the lowest division rates, from yeast extract into whole yeast the next highest, while the highest are those from yeast extract into yeast extract.

The chemically treated yeasts B33413 and B33425 contrast with B13412 (plain finely powdered whole yeast) in that the addition of dextrose improves the former and harms the latter (Experiments 3 to 15). Yeast B33425 also contrasts with B13412 in that fractional sterilization (85° C.) does not help, while marked improvement is noted for the latter (Experiments 8, 9, and 13, 14).

Finally, increasing the complexity of the medium beyond that of Experiment 18 results in no gain. The liver used in Experiment 21 is

[5] Culture media containing dextrose turn strongly acid after inoculation of *Glaucoma pyriformis*, even in the presence of considerable phosphate buffer. The flasks containing dextrose (Experiments 2, 3, 4, 6, 7, 8, 11, 12, 13, 20, and 21) also contained excess $CaCO_3$, which maintained the hydrogen ion concentration within the limits of pH 7.30 to 6.83.

TABLE I

Growth of Glaucoma in different culture media. Fifty cc. of medium in 125-cc. flasks in all experiments. Inoculum 15 = 1,490 glaucomas, 7 = 717. Counts taken after 24 hours except for wheat which represents 72 hours of growth. *F. st.* indicates that the medium was fractionally sterilized.

Experiment	Medium	Inoculum	Yield	Mean division rate
1	5% Wheat	15	870,000	3.06 (3rd day)
2	0.5% Yeast (Fleischmann) 0.5% Dextrose	(many counts)		6.02
3	0.5% Yeast No. B33413 0.5% Dextrose	15	143,200	6.60
4	0.5% Yeast No. B33413 0.5% Dextrose	7	64,900	6.49
5	0.5% Yeast No. B33413	7	12,740	4.15
6	0.5% Yeast No. B33425 0.5% Dextrose	15	125,100	6.39
7	0.5% Yeast No. B33425 0.5% Dextrose	7	12,080	4.07
8	0.5% Yeast No. B33425 (*F. st.*) 0.5% Dextrose	7	13,300	4.21
9	0.5% Yeast No. B33425 (*F. st.*)	7	11,630	4.02
10	0.5% Yeast No. B33425	7	25,500	5.15
11	0.5% Yeast No. B13412 0.5% Dextrose	15	116,300	6.29
12	0.5% Yeast No. B13412 0.5% Dextrose	7	46,600	6.02
13	0.5% Yeast No. B13412 (*F. st.*) 0.5% Dextrose	7	71,000	6.63
14	0.5% Yeast No. B13412 (*F. st.*)	7	118,300	7.37
15	0.5% Yeast No. B13412	7	57,000	6.32
16	0.5% Yeast Extract 0.1% Yeast No. B33413	15	177,500 202,000	6.99

TABLE I (*Continued*)

Experiment	Medium	Inoculum	Yield	Mean division rate
17	0.5% Yeast Extract 0.1% Yeast No. B33425	15	213,700 211,500	7.16
18	0.5% Yeast Extract 0.1% Yeast No. B13412	7	68,500 69,200	6.59
19	0.2% Yeast Extract 0.2% Peptone 0.1% Yeast No. B13412 0.1% Kidney No. 23211	15	183,000 169,000 173,000	6.87
20	0.2% Yeast Extract 0.2% Peptone 0.1% Yeast No. B13412 0.1% Kidney No. 23211 0.4% Dextrose	15	152,000 149,000 155,300	6.70
21	0.2% Yeast Extract 0.2% Peptone 0.1% Yeast No. B13412 0.1% Kidney No. 23211 0.2% Dextrose 0.2% Eli Lilly Liver (unheated)	15	156,400 139,000 131,000	6.58

Eli Lilly Company's Extract No. 343, sterilized by passing through a Seitz bacteriological filter.

Hydrogen Ion Concentration

The reaction of the Difco yeast extract (prepared in Peters' medium) is pH 6.91–6.95, of the complete medium after autoclaving, 7.00–7.05, and after supporting growth of large numbers of *Glaucoma* for 24 hours, 7.00–6.97, as measured with a quinhydrone electrode.

Repeated efforts to duplicate Elliott's (1933) bimaximal growth in the regions of pH 6 and 7.5 with a low point at 7, failed. At pH values of 6.7 and 7.4 it was impossible to equal the yields of medium regulated nearer neutrality. Slow growth, finally yielding large numbers, was achieved at the following extremes: pH 4.35 and pH 9.37 $\pm$ .05.

Growth-promoting Characteristics of the Yeast

The apparent presence of an unstable growth-promoting substance in the fractionally sterilized yeast No. B13412 (Table I, Experiments 13 and 14) suggested some more tests, which are recorded in Table II.

Experiments from inocula 844 and 1,696 were run simultaneously with those of Table III and are therefore strictly comparable. Even when the yeast is 0.1 per cent, half the concentration of the standard medium (p. 431), marked acceleration is evident.

The maximum rates of growth here recorded are not very high when compared with those of Enriques (1916). Working at a higher temperature and in the presence of bacteria he obtained as many as 13 generations in 24 hours, and never less than 9.

Growth from Inocula of 0.2 cc. Containing Varying Numbers of Glaucoma

The volume 0.2 cc. was chosen simply because of its convenience. Table III gives unselected results of several experiments taken at

Table II

Growth of *Glaucoma* on varying concentrations of fractionally sterilized (85° C.) whole yeast No. B13412. All media contain 0.5 per cent yeast extract. To be compared with corresponding inocula in Table III.

Whole yeast	Inoculum	Yield	Division rate
0.1%	844	242,000	8.16
0.1%	844	248,000	8.20
0.2%	1,696	617,000	8.50
0.2%	1,696	628,000	8.53
0.4%	1,330	549,600	8.69
0.4%	1,330	507,000	8.57

different times and on different batches of medium. A comparison of the division rates resulting from inocula 581 to 1,696 indicates that average rate of growth is about constant over this range. When 4,270 are inoculated a distinct drop in rate of increase occurs, while 26,640 give only about 72 per cent as rapid growth. This is presumably a consequence of crowding.

Inocula between 500 and 2,000 per 0.2 cc. are very readily obtained either from flasks which have been subject to daily sub-culture using a 0.2 cc. transfer volume, or from a two-day culture inoculated with one ciliate. Inocula below 581 were not tested because it is difficult to count the number of ciliates in such dilute cultures.

These results were unselected. There is every reason to believe that, with repetition of one kind of experiment using medium made up in large quantities, greater uniformity of yield could be achieved.

Growth from an Inoculum of One Dividing Glaucoma

A dividing ciliate is a convenient inoculum because the age is known within a few minutes. It is here treated as an inoculum of two glaucomas. It was found that two days of growth gave numbers which could be accurately counted.

Table III

Relation between number of glaucomas inoculated and rate of growth. Volume of inoculum = 0.2 cc. Period of growth = 24 hours.

Inoculum glaucomas in 0.2 cc.	Yield glaucomas in 50 cc.	Division rate
581	146,000	7.97
	140,500	7.94
	138,500	7.90
	137,000	7.89
844	201,100	7.89
	204,900	7.92
	209,100	7.95
962	227,000	7.89
	228,000	7.89
	238,200	8.02
	241,800	8.02
1,696	417,700	7.95
	416,800	7.94
	407,800	7.91
	Mean	7.93
4,270	963,000	7.82
	968,000	7.83
26,640	1,483,000	5.80
	1,575,000	5.88
	1,560,000	5.87
	1,499,000	5.82

Table IV gives counts after 2 days and again after 3 days. Considering the 48-hour counts first, two differences are evident when compared with Table III, inocula 581 to 1,696. First, the mean division rate is lower, although continuity occurs at the upper extreme. Second, greater variability in division rates is evident. The 48-hour experiment was repeated with closely similar results, giving a mean division rate of 7.76. The mean division rate during the third day is 4.80. The uniformity of division rates for the 72-hour interval probably reflects a consequence of crowding.

Comparing inoculum 26,640 of Table III with the 48-hour yield of Table IV, it is seen that the latter is 2.5 times the former. The mean yields, on the other hand, differ by only 17 per cent (1,529,250–1,838,600). This suggests that these populations are practically static; i.e., they represent the "crop" for this particular strain of *Glaucoma pyriformis*. They are still very healthy organisms.

The Interval Required for Division

What is the actual behaviour of individuals in these flasks? Does each divide after a certain interval in a regular fashion, or are the cultures mixtures of degenerating and more or less rapidly multiplying ciliates? To answer qualitatively first, extensive observation of flask-cultured glaucomas under the microscope never revealed degenerating

Table IV

Growth from an inoculum of one dividing glaucoma after 2 days and after 3 days.

48 hours		72 hours	
Yield	Division rate	Yield	Division rate
57,200	7.65	1,691,000	6.73
60,700	7.69	1,943,000	6.80
67,600	7.77	1,849,000	6.77
66,500	7.76	1,878,000	6.78
78,900	7.88	1,832,000	6.77
Mean	7.75	Mean	6.77
		Mean for last day	4.80

or conjugating forms; on the other hand, uniformity in appearance was a striking characteristic. When this strain was cultivated on Fleischmann's yeast (p. 430), a considerable proportion of the population always appeared to be conjugating. Closer inspection revealed two apposed longitudinal depressions which apparently developed some hours after fission. Such forms nourished and divided like the others. All traces of this phenomenon disappeared in two transfers on the Difco medium.

The following experiments were carried out in Columbia isolation dishes enclosed in Petri dishes. One hundred or so glaucomas were removed from a young and thriving flask culture. put in a dish containing 1 cc. of medium, and watched through the Petri cover. When one was seen to be well along in division, it was removed with a pipette (medicine dropper) of generous bore to a similar dish containing

1 cc. of medium, and the time of separation noted. A series of such experiments was watched continuously at a temperature of 24.2° to 25.2° C. to determine the time required for the next fission.

A surprising result was obtained. Irregularity between the limits of 2 hours and 45 minutes for the shortest interval to 4 hours and 25 minutes for the longest, was observed. Daughter cells were as irregular as any others. The mean was roughly 3 hours and 25 minutes (3.42 hours). To give a mean division rate of 7.93 a division every 3.028 hours is necessary. The second division was also followed [6] but no significant difference in behaviour occurred.

Early Growth of Glaucoma Cultured in Flasks

If the marked irregularity and slowness of growth of organisms observed in shallow dishes is not due to incidental technical features of the experiment, it should be possible to demonstrate the same thing in flask cultures by counting before large numbers make their appearance.

TABLE V

Growth during the first 9-hour period compared with later growth. Inoculum = 1,696 glaucomas in 0.2 cc.

Growth during 9 hours		Growth during 24 hours	
Yield	Division rate	Yield	Division rate
11,470	7.35	417,700	7.94
10,710	7.11	416,800	7.94
9,965	6.82	407,800	7.91
Mean	7.09	Mean	7.93
Mean for last 15 hours			8.44

Table V gives results from an inoculum of 1,696 ciliates after only 9 hours, then again after 24 hours. The mean division rate for the first 9 hours is 7.09, lower than that for 24 hours, and gives a mean interval required for division of 3.385 hours. This agrees satisfactorily with the mean interval of 3.42 actually observed in the previous section. The mean interval required for division during the last 15 hours is 2.84 hours.

An objection could be raised to this experiment because the inoculum was from a culture containing large numbers of ciliates,

[6] Ciliates other than the two under observation were removed; hence the second interval followed without manipulation by pipette.

8,480 per cc. (424,000), and might be "conditioned," so the shock of transfer to fresh medium would explain the early retardation.

Accordingly, in spite of the labor involved in counting small concentrations of ciliates, an inoculum of 0.5 cc. containing only 661 glaucomas was tried. Counts were taken after 6 hours, 12 hours, and 24 hours.

Table VI substantiates Table V in that a definite acceleration in rate of growth occurs during the 24-hour period. Since the six-hour rate (7.27) is higher than that for 9 hours (7.09), it is possible, as suggested above, that some shock of transfer plays a small rôle in the experiments of Table V. The mean rate of 8.55 for the last 12 hours gives an interval required for division of 2.8 hours: the glaucomas divide every 2 hours and 48 minutes.

TABLE VI

Growth during the first 6-hour period compared with later growth. Inoculum = 661 glaucomas in 0.5 cc.

Growth during 6 hours		Growth during 12 hours		Growth during 24 hours	
Yield	Division rate	Yield	Division rate	Yield	Division rate
2,497	7.67	8,540	7.38	166,000	7.97
2,331	7.27	8,660	7.42	168,500	8.00
2,163	6.84	8,760	7.45	168,000	7.99
Mean	7.27	Mean	7.41	Mean	7.98
Mean for second 6 hours			7.57		
Mean for last 12 hours					8.55
Mean for last 18 hours					8.23

The division rate for the first 6 hours gives an interval required for division of 3.30 hours. This value and that for 9 hours in Table V (3.385) agree satisfactorily with the mean, 3.42, of intervals actually observed in the preceding section.

The probable error of the 6-hour counts corresponds in terms of division rate to about 0.25, and of the 9-hour counts of Table V to about 0.2. The recorded order corresponds to the order in which the flasks were inoculated without refilling the pipette. Accordingly, the serial variability observed in the 6-hour counts and also in the 9-hour counts indicates actual difference in growth, and can be explained by assuming a loss of 30 to 50 ciliates on the side of the pipette at each inoculation.

The apparent lag in early growth recorded in Tables V and VI suggests the "allelocatalytic effect" of Robertson (Johnson, 1933, gives a good recent review of this subject). Definite conclusions concerning the nature of this lag in *Glaucoma* must await a technique which will permit accurate counting of small numbers.

Comparison with Other Strains of Glaucoma pyriformis

Ten different strains of *Glaucoma pyriformis* have been either isolated in this laboratory or sent here through the courtesy of other investigators. It is possible to distinguish between a few of them morphologically, and between every one of them by simple counts of growth in the yeast medium.

In spite of the fact that this medium was developed specifically for my yeast-requiring strain, three other strains, including Dr. Elliott's "*Colpidium striatum*" (1933), which resembles Lwoff's strain in that it grows luxuriantly on peptone, grow better on it. The latter divides about 1.05 times as fast as mine. The most difficult strain to distinguish from mine is Dr. Butterfield's (1929) "*Colpidium*," which is interesting in that it will grow for a few transfers in peptone, then fails to increase. It divides about 1.03 times as fast as mine. This form is thus intermediate in its nutritive requirements between the more typical peptone-growing strain and the yeast-requiring variant described in this paper. The other strains divide less rapidly, the lowest division rate recorded being 6.23, about 0.84 times as fast.

Morphologically, a slender ("schlanke form") strain is the most striking variant: it is distinctive even when very well nourished. The author is acquainted with this strain through the courtesy of Dr. Elliott and Dr. Kahl.

Glaser and Coria (1935) obtained from hot springs strains of *Saprophilus oviformis* (= *Glaucoma pyriformis*) which grow vigorously at 37° C.

Summary

1. The strain of *Glaucoma pyriformis* reported upon in this paper will not grow in peptone in pure culture, or in this plus a fine suspension of insoluble particles of extracted yeast.

Yeast extract (Difco), particle-free, supports a division rate of 6.86.

Whole yeast (Difco—autoclaved) plus either peptone or yeast extract supports division rates from 7.65 to 8.02.

Media more complex than this do not increase the division rate.

If the whole yeast added to the yeast extract is fractionally sterilized rather than autoclaved, marked increase in division rate results.

2. There is but one optimum hydrogen ion concentration. This is in the region of pH 7.

3. When 600 to 2,000 glaucomas are inoculated in 50 cc. of medium, the division rate is 7.89 to 8.02 for the first day.

When larger numbers are inoculated the division rate is lower.

4. If one dividing glaucoma is inoculated, the mean division rate (48 hours) varies from 7.65 to 7.88.

5. Observation of isolated organisms in shallow dishes revealed marked irregularity in the interval required for division: from 2 hours and 45 minutes for the shortest interval to 4 hours and 25 minutes for the longest. The mean was 3 hours and 25 minutes, corresponding to a division rate of 7.

6. Counts of growth in less than 24 hours apparently demonstrate a lag in early growth. No definite conclusions were drawn because of the large error resulting from counting low concentrations of ciliates.

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REGENERATION IN THE POLYCHÆTE CLYMENELLA TORQUATA

III. Effect of Level of Cut on Type of New Structures in Anterior Regeneration

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Although many polychæte annelids regenerate posteriorly, relatively few readily form new heads. The succession of events in anterior regeneration in *Clymenella torquata* after the loss of 5 segments has already been described (Sayles, 1932). At that level regeneration is usually complete after about fifteen days. During the summers of 1929–31 a record was kept of regenerates among worms as they were brought into the laboratory. Of 1,364 specimens of *Clymenella*, 33 were regenerating anteriorly, none of these at levels posterior to the sixth segment. Under laboratory conditions it has been possible to secure a number of regenerates at the tenth segment and some even at the eleventh.

In this paper anterior regenerates at various levels are discussed. These were obtained during work carried out at Woods Hole from 1928 to 1934, inclusive. The materials and methods have already been discussed in a paper on posterior regeneration in this worm (Sayles, 1934).

Experimental Results

Anterior regeneration after the removal of from *1 to 6 segments* may be considered first. Upon cutting at these six levels the mortality of posterior pieces was relatively low, 85 per cent or more of the pieces surviving for 10 days or longer. After the loss of one or two segments a complete bud was formed by the tenth day. This was also usually the case after the loss of three segments. At the fourth and fifth segments the majority of buds were complete after ten days but about one-third of the pieces required 11 to 15 days to form a bud which showed complete external segmentation, parapodia and folds associated with the peristomium. At the sixth most of the pieces were just beginning to show external evidences of segmentation after 10 days and, in most cases, they were complete, as far as general external features were concerned, by the fifteenth day.

Of the specimens cut at the *seventh segment* about 70 per cent survived. Here, as at the sixth, organization of the new bud was slower than at more anterior levels. In addition one piece failed to produce a complete bud of the usual shape. Instead there was formed a peristomium of ordinary size and structure but followed by five, possibly six, segments of much smaller diameter. This specimen was observed early in this work (in 1928) and unfortunately it was neither carefully drawn nor preserved for further study. This bud was probably of the type which has appeared on regenerates at the eighth segment and somewhat more posterior levels. All other pieces at this level which survived for 15 days had produced complete new buds by that time.

Three regenerates at the eighth segment. Drawn with aid of camera lucida.

FIG. 1. Ventro-lateral view of weakly segmented bud after 12 days. × 16.
FIG. 2. Right-lateral view of regenerate, at 21 days, which had probably broken off at a very slender region in the middle of a bud. × 13.
FIG. 3. Right-lateral view of a somewhat crude bud after 18 days. × 16.

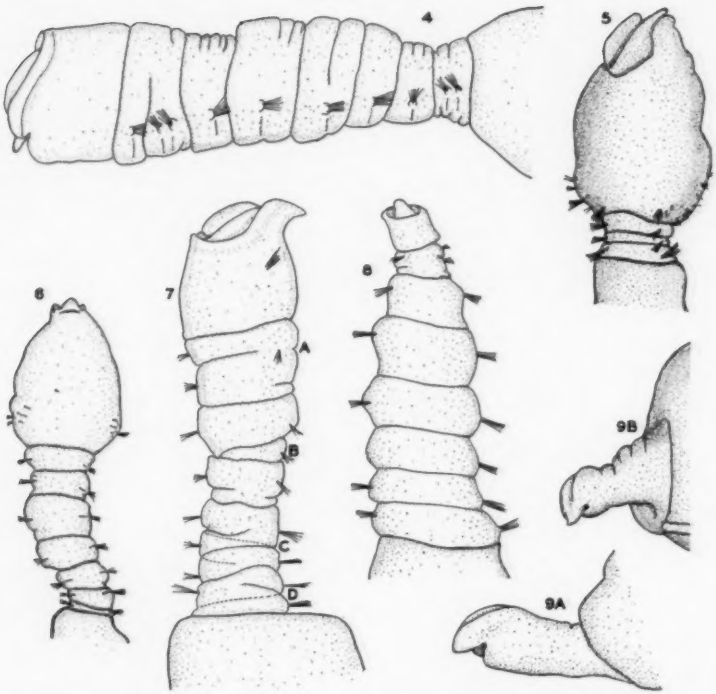
At the level of the *eighth segment* about 30 per cent of the pieces cut were dead after 10 days. Of the 24 survivors 21 had small buds which were either non-segmented or only weakly segmented (Fig. 1). Two others had buds consisting of one or two distinct segments of good size, anterior to which was a small unsegmented region (Fig. 2). In these two pieces it might seem that the proximal segments have become established without the development of an organized peristomial region. A study of anterior regenerates at more posterior levels, however, suggests that in each of these two cases a bud may have been formed with a very slender mid-portion. Buds of this latter type have been observed at the ninth, tenth and eleventh segments. Some of these have been broken although considerable care was taken to keep them intact. The portion of the bud then remaining attached to the old part was similar in appearance to the arrangement of the new tissue observed in these two specimens at the

eighth segment. In the case of the remaining piece, regeneration progressed very slowly. The bulb of gut, formed in wound closure, did not disappear until the third day and no new tissue was apparent until the seventh. On the tenth day only a very small bud had been formed. After 18 days this worm had produced a complete, well-formed bud. Three of the others had died. Of the latter two were the ones which had probably been broken previous to the tenth day. At this time 15 had formed complete buds consisting of a head region followed by seven setigerous segments of medium diameter. Another piece had a bud consisting of a well-developed head region followed by a slender part of indistinct segmentation. This worm died on the twenty-third day without showing any marked change in the meantime. Two others had heads of the type shown in Fig. 3. The other two were of the broken type described above, but the result of recent breaks.

At the eighth segment, therefore, the majority of survivors replaced accurately the segments lost. Some, however, produced very slender buds but none of these pieces survived long enough to complete segmentation. A few produced buds which were indistinctly segmented and had but small head folds.

At the *ninth segment* about 70 per cent of the pieces survived ten days. After twenty days 37 out of 77 were alive. These 37 may be placed in 8 groups according to the type of bud formed. (1) Nine had formed complete buds of 8 setigerous segments plus an asetigerous head region, all of these of the usual shape and size. (2) Four had produced buds having the full number of segments, but with the third to eighth segments, inclusive, of a diameter much smaller than that of the first two. (3) Each of 4 others had a well-developed head region followed by a slender part. The anterior portion of the latter showed about four setigerous segments and the posterior part, near the original cut piece, was indistinctly segmented. (4) Seven had short buds of medium diameter with head regions of the normal type. One consisted of 6 segments and one of 4 segments. Each of the other 5 had a peristomium and 3 or 4 setigerous segments followed by a weakly segmented region with no parapodia visible. (5) One consisted of 10 segments the last 3 of which were a little irregular. (6) Three had short conical buds without any constrictions apparent. (7) One had not regenerated beyond wound closure. (8) Eight had buds of various shapes with peculiar arrangements of both parapodia and segmental constrictions.

Several of the last-named group merit more detailed descriptions. One of them (Fig. 4) had a bud which was divided externally into nine



Seven regenerates at ninth segment. Drawn with aid of camera lucida.

FIG. 4. Bud of good shape except for extra pairs of parapodia on third and ninth segments. Twenty-nine days. Lateral view. × 10.

FIG. 5. A bud with proximal portion segmented and distal part swollen and unsegmented. The number of setæ in each group on the swollen region is accurately shown. Twenty-eight days. Dorsal view. × 10.

FIG. 6. Another bud with distal part swollen and unsegmented. Twenty-nine days. Ventral view. × 6.

FIG. 7. Bud possessing split-segment (*A*), intercalated segment (*B*), left-hand spiral of one and one-half segments (*C*), and right-hand spiral of a little more than one-half segment (*D*). Ventral positions of intersegmental grooves, where they do not match the dorsal ones, are shown in dotted lines. Twenty-eight days. Dorsal view, somewhat lateral in anterior part. × 10.

FIG. 8. Another bud showing irregularities confined mostly to distal part of bud. There was no left parapodium on what was probably the third segment. The notopodium (but not the neuropodium) of the seventh segment was missing. Twenty-nine days. Dorsal view. × 10.

FIG. 9. Two somewhat weakly developed buds. *A*. On the tenth day. Left lateral view. × 16. *B*. On the twelfth day. Left ventro-lateral view. × 12.

segments, the first of which was asetigerous and of ordinary shape. A peculiar thing about this bud was that the third and ninth segments each had two pairs of parapodia. Both neuropodial and notopodial portions were present. Aside from this point the bud was normal in appearance. If, on microscopic examination, it is found that each of these double regions belongs really to 2 segments, it is clear that the piece has formed 11 segments after the loss of 9. If not, then there has at least been a duplication of parts. It is also interesting to note that these unusual regions were some distance from one another, one next to the old part and the other near the distal end of the bud. Another of these pieces (Fig. 5) consisted of three proximal segments, apparently of the usual type, in front of which was a somewhat swollen unsegmented region, the anterior part of which bore considerable resemblance to the usual type of first segment. The posterior part of this region bore a number of clusters of setæ, none of which were of the neuropodial type. Three of these clusters were of the same appearance as ordinary notopodia but the others consisted of only 2–4 setæ each. Here, then, there is a case of completion of the three proximal segments, and, for the most part, of the peristomial segment. In between, however, was a region where external segmentation and parapodium-formation had proceeded in an unusual manner. Another of these peculiar regenerates had formed a bud consisting of a somewhat swollen anterior part followed by a slender region composed of seven segments, each provided with neuropodia and notopodia of the ordinary type (Fig. 6). The portion of larger diameter was unsegmented. At its anterior tip it had weakly-developed peristomial folds. Near its posterior end it bore several groups of setæ. On the right side there were two notopodia, ventral to which were three vertical rows of uncini, the central row being longer and bearing uncini larger than either of the others. On the left side there was but one notopodium, but here also there were three vertical rows of uncini all of about the same size. Here, then, we have a case where organization of the posterior two-thirds of the bud had gone on in the usual manner but that of the anterior part had been abnormal.

A fourth individual (Fig. 7) in this same group consisted of a peristomium, bearing a pair of parapodia, and the apparent equivalent of 8 additional setigerous segments. The latter, however, showed several peculiarities of arrangement. Immediately behind the peristomium was a region consisting of a single segment on the right but split on the left to form two parts. Next there was a regular segment. Then a separate half segment was present on the right side. Behind this was another regular segment which was followed by a spiral

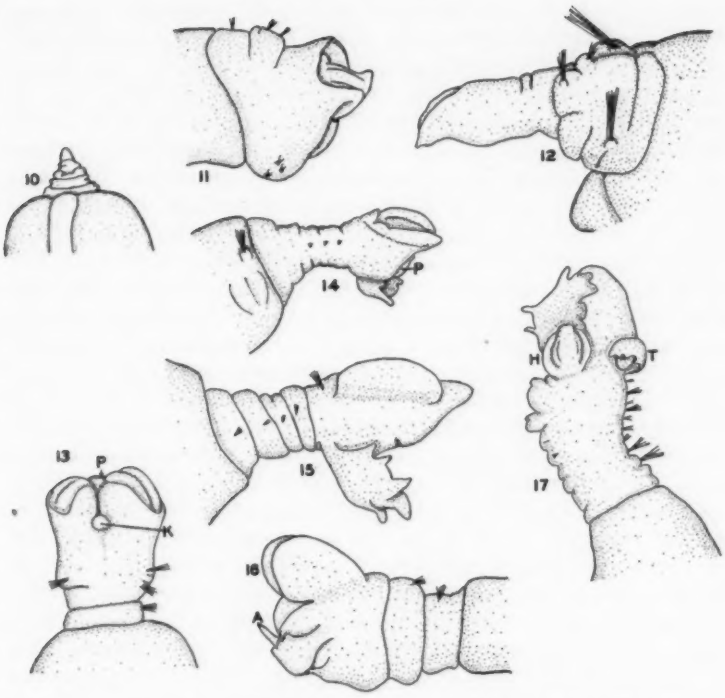
segment beginning on the dorsal side and running to the left one and one-half turns to end on the ventral side. Behind this was another spiral region running in the opposite direction and apparently ending laterally on the left side. It was very difficult to determine the exact limit of this adjacent to the old part of the worm. Although this last region might possibly be interpreted as an intercalated half-segment, it has more the appearance of a very short spiral. The fifth and last one of this group to be described is shown in Fig. 8. The posterior two-thirds of the newly formed bud was of the usual diameter. The remaining portion was of about one-half this size. The larger posterior portion of the bud consisted of five setigerous segments with only one point that was unusual, namely, the absence of the left notopodium from one segment although the neuropodium was present. Anterior to these five segments was another complete setigerous segment, of intermediate size. The smaller anterior part of the bud consisted of two main divisions. The anterior one was clearly a peristomial region. The prostomium was, in proportion, shorter and thicker than usual. The head folds on the peristomium were lower and broader than those ordinarily found in this region. The posterior division of this smaller region was incompletely marked off into three parts, the anterior of which bore a parapodium on each side. The middle one of these small parts bore a parapodium on the right side only; the posterior one was without parapodia. In this case, also, the region near the old part was organized in the usual manner and the anterior part of the bud, farther away from the old piece, underwent an unusual organization.

Posterior pieces regenerating anteriorly at the *tenth segment* were much more susceptible to infection than pieces cut at more anterior levels. Of 195 pieces cut only 83 (or about 43 per cent) survived for ten days. At this time, 35 had formed buds of about the type shown in Fig. 9. Thirty-six had produced buds which were either very short or had irregular shapes. Ten showed no external evidence of new tissue. One was slightly irregular with some resemblance to a small heteromorphic tail. The other survivor had formed a bud, the main part of which did not differ greatly from Fig. 9. From the right side of this main part there projected a somewhat lobulate structure arising at a level slightly posterior to that of the mouth. Unfortunately, this specimen died on the fourteenth day before there had been time for many noticeable changes from this condition. At this time, it was similar in appearance to the early stages in the formation of multiple structures such as those described below.

At the end of 20 days there were 55 survivors, which might be

placed in 5 groups. (1) Two had small buds in the early stages of head formation and segmentation. Both of these died at about this time without undergoing any marked changes. (2) Five showed no external evidence of new tissue. It should be noted, however, that one of these had a slender bud at 10 days. Apparently this bud was lost and not replaced, possibly because of insufficient time. These were in the same condition when they were discarded or died after 20–24 days. (3) Four had produced irregular buds. One of the latter, a short weakly segmented bud, was drawn (Fig. 10) and killed at this time. Another (Fig. 11) had a short bud somewhat bloated in appearance and deep red in color. It had fairly well formed head folds and prostomium, but showed practically no evidence of segmentation. On each side were 3 notopodia, rather irregularly arranged especially on the right side. The third of these buds was short and conical. Nine days later this bud (Fig. 12) consisted of a short, broad proximal portion with two pairs of parapodia and a distal part similar in appearance to a typical head bud of about ten to twelve days. This may possibly have been a re-regenerate. The last of these buds was a peristomium followed by a short unsegmented region. On this bud were several extra knob-like growths which looked like the beginnings of multiple structures. Unfortunately this animal died at this time and before any drawings of it had been made.

(4) Six others of the survivors at 20 days had multiple structures. One consisted of a single, weakly segmented bud with a mouth which was practically terminal. The dorsal side of the peristomium was double with two complete parts, each consisting of head folds enclosing a prostomium. This double appearance was first unquestionable on the twentieth day. By the following morning the animal had died. No drawing is available. Another bud (Fig. 13) consisted of 3 setigerous segments and a double peristomial region with its parts so turned that the sides ordinarily ventral were more or less facing one another. Between the latter there was a single mouth with a small proboscis exhibiting the usual activity. Three of the other buds were similar in that each was a well-developed bud of several segments with a peristomium of quite good shape and with the second part a heteromorphic tail (Figs. 14, 15 and 16). There can be no question that these extra parts were tail buds. In the first place, the cirri, so characteristic of the anal collar of a normal worm, were present. Also these extra buds were almost identical in appearance with some of the unusual tails produced in posterior regeneration at the eighth to tenth segments. In different posterior regenerates at these levels many intermediate forms between these odd types and the usual one have



Eight regenerates at the tenth segment. Drawn with aid of camera lucida.

FIG. 10. Ventral view of a weakly developed bud. The segmentation was very indistinct on dorsal side. No parapodia were visible. Twenty days. × 12.

FIG. 11. Right dorso-lateral view of short, swollen irregularly segmented bud of about 4 segments. Twenty-five days. × 12.

FIG. 12. Left view of a bud irregular in portion near old part. Twenty-nine days. × 24.

FIG. 13. Bud with anterior part of peristomium double. *P*, proboscis. *K*, knob of tissue. Twenty days. × 22.

FIG. 14. Right view of regenerate with ventral tail outgrowth. *P*, proboscis. Twenty days. × 10.

FIG. 15. Right view of regenerate with ventral tail outgrowth. Twenty-six days. × 16.

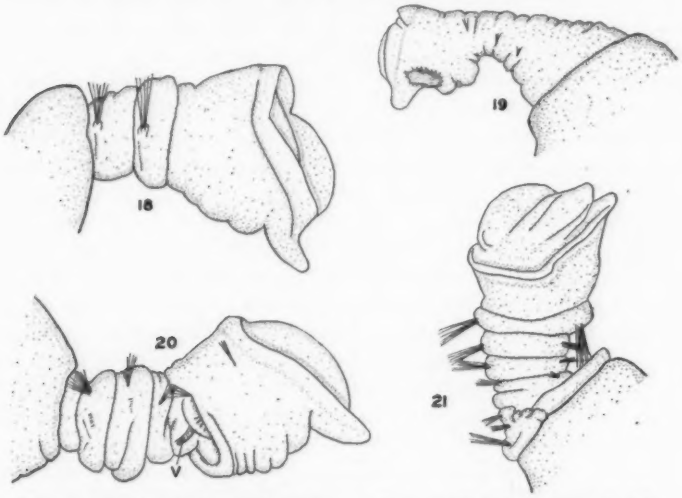
FIG. 16. Left view of regenerate with tail outgrowth on left side. *A*, anal cone. Twenty-six days. × 16.

FIG. 17. Dorsal view of heteromorphic tail with small extra head (*H*) and tail (*T*) outgrowths. Twenty days. × 16.

been found (Sayles, 1934). In two cases the heteromorphic tails arose from the mid-ventral region somewhat behind the mouth. In the third instance the small tail-bud arose mid-laterally at approximately the same longitudinal level as the mouth. This point of origin matches the position of the circumœsophageal connective. The last of these buds was the one which, on the tenth day, resembled a heteromorphic tail. By the thirteenth day this bud clearly consisted of several rather irregular segments terminating in an incomplete anal collar, open on the left side and bearing 6 cirri. At this time there were present in the vicinity of the base of the collar two rather large outgrowths without any apparent differentiation into head or tail type. These two extra parts gradually assumed distinct shapes until after 20 days the condition shown in Fig. 17 existed. It may be noted that at this time there were at least 9 new segments anterior to which was the multiple region. The main terminal part was an anal segment bearing a half-collar on which were 7 cirri. From the right dorsolateral region at the base of this terminal segment arose a small bud including a partial anal collar with about five cirri and also a long anal cone. In the vicinity of the dorsal end of the open anal collar and somewhat to the left of the small extra anal segment there was the other small bud which showed the main distinguishing characteristics of a head segment. An interesting point is that this segment was in the proper position with relation to the old part despite the fact that it did not appear until after the main heteromorphic tail was clearly organized.

(5) The remaining 38 individuals alive at this time all had peristomial regions of ordinary size and shape. There was, however, considerable variation in the setigerous regions. For instance, one bud had two post-peristomial segments with no evidence of setæ. Two others had two such segments but with parapodia (Fig. 18). Three had a peristomium bearing a pair of parapodia at the level of the posterior end of the peristomial crown; 2 of these had 5 other setigerous segments and one had 4. Ten others had regular setigerous segments varying in number between 5 and 9. Two others had a number of segments but only 3 (Fig. 19) and 4, respectively, with parapodia. Each of these 18 buds was of fairly uniform diameter throughout its entire length. One of the other 20 consisted of the peristomium followed by 6 setigerous segments. The right side of the second setigerous segment was swollen and bore 2 extra parapodia. The remaining 19 in this group had slender proximal regions, 17 of them with regularly formed setigerous segments varying in number from 3 to 8. One of the others (Fig. 20) consisted of 6 or 7 segments

of which 4 had parapodia. In addition there was a pair of clusters of notopodial setæ far forward on what appeared to be a part of the peristomium. The proximal part of the bud was very irregular. The last of this group (Fig. 21) had a well-formed peristomium followed by 4 setigerous segments. Posterior to these there was an asetigerous region. Between the latter and the old part, on the left side only, there was an irregular structure bearing 3 notopodia. As a result the bud as a whole was tipped to the right.



Four regenerates at the tenth segment. Drawn with aid of camera lucida.

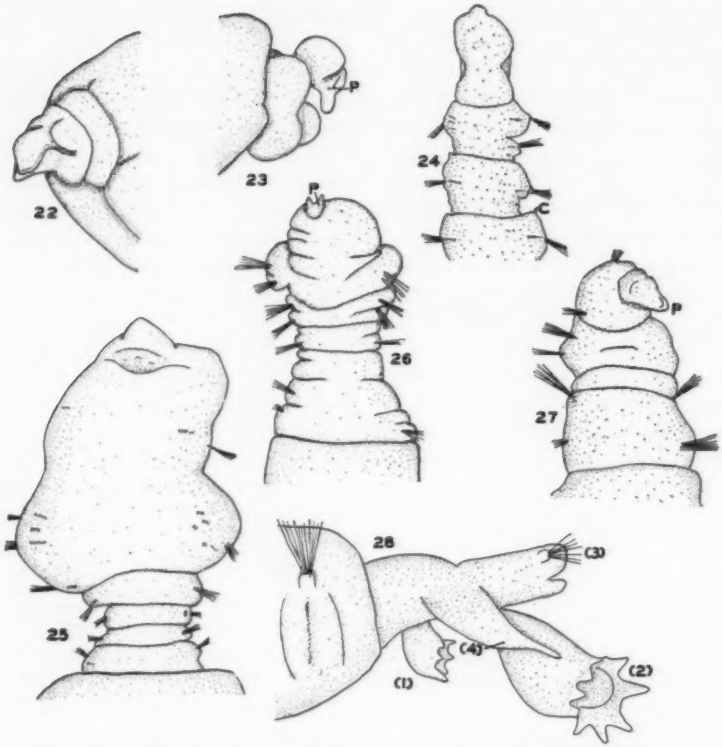
FIG. 18. Three-segment bud of good shape. Twenty-eight days. × 16.
FIG. 19. Left view of a bud at 27 days. × 12.
FIG. 20. Right ventro-lateral view of a somewhat irregular bud. *V*, ventral blood vessel. Twenty-three days. × 16.
FIG. 21. Dorsal view of regenerate with region bearing 3 notopodia intercalated on left at base of bud. Twenty-eight days. × 16.

At the *eleventh segment*, of 113 pieces cut, 30 (about 26 per cent) survived for 10 days. Of these, 11 showed no external evidence of new tissue. Four had short, unsegmented buds with but small headfolds. One had a double structure consisting of a heteromorphic tail from the ventral side of which another, smaller tail had grown out. One had a short heteromorphic tail terminating in an anal collar which was open ventrally and possessed 5 or 6 anal cirri. The other 13 had irregular buds of various types. Two of these were killed and 14 others died during the next 10 days. One of those killed (Fig. 22)

had a bud composed of a somewhat irregular peristomium followed by a single asetigerous segment.

Of the 14 survivors at 20 days, 2 were without new tissue. Neither of these changed in appearance up to the times of death, one at 27 days, the other at 30. On the thirteenth day the single heteromorphic tail had dropped off. Little if any further new tissue was formed by this piece up to the time of its death on the thirtieth day. Four others had buds with peristomial regions of fairly definite shape followed by slender regions of 4–7 setigerous segments of quite regular arrangement. One worm had a bud (Fig. 23) made up of several irregular parts, the most anterior of which was apparently a much misshapen peristomial region.

Five other buds were 3–6 mm. long but irregular. Four of these survived until the twenty-eighth to the thirty-second day at which time they were drawn and killed. One, the anterior part of which is shown in Fig. 24, had a peristomial region followed by 8 setigerous segments, 2 less than were removed. The peristomium was of fair shape but with no trace of a mouth. It was followed by a region including at least two segments (which we shall call the second and third). These were separated from one another on the left but not on the right. It may be noted that there was an irregular arrangement of parapodial parts here. The fourth and fifth segments were of usual appearance, including the collar characteristic of the fifth segment of this species. Clearly then the 2 missing segments of this bud must have been omitted from its posterior part. The second of these buds (Fig. 25) included a posterior part of 4 setigerous segments anterior to which there was a swollen region a little over 2 mm. long and terminating in a peristomium of very nearly the usual appearance. This swollen region bore a number of groups of setæ of irregular arrangement. The third of these buds (Fig. 26) varied in diameter in different regions. The anterior part of the bud was without setæ but was constricted in several places. At its anterior tip was a very small knob of tissue, notched anteriorly. Aside from this knob, which might possibly be considered as a dwarfed, irregular peristomium, none of the characteristic head-features was present. The remainder of the bud was somewhat indefinitely marked off into segments. Judging on the basis of notopodia and pronounced constrictions, 7 or 8 segments were present in this part. The last of these buds consisted of 8 setigerous segments gradually reducing in size from the old part toward the tip. The anterior half of this bud is shown in Fig. 27. Anterior to the setigerous segments was a small region twisted somewhat dorsally. The latter looked like a minute, early peristomium.



Seven regenerates at the eleventh segment. All except Fig. 28 drawn with aid of camera lucida.

FIG. 22. Two-segment bud with somewhat irregular peristomium. Fifteen days. × 16.

FIG. 23. Right view of irregular bud probably including a peristomium (*P*). Nineteen days. × 16.

FIG. 24. Ventral view of anterior part of a bud which lacked a mouth. *C*, collar on fifth segment. Twenty-eight days. × 16.

FIG. 25. Ventral view of a bud, the anterior part of which was swollen and unsegmented but possessing a number of groups of uncini as well as several notopodia. Twenty-eight days. × 16.

FIG. 26. Dorsal view of a regenerate of 28 days. *P*, possibly a dwarfed, irregular peristomium. × 16.

FIG. 27. Dorsal view of anterior part of bud which included three more segments. First two divisions twisted so that peristomium (*P*) is hooked dorsally. Thirty-six days. × 22.

FIG. 28. Right lateral view of a multiple bud at 22 days. The original main heteromorphic tail (2) was tipped somewhat ventrally by the development of two outgrowths—(3) and (4)—on dorsal side of bud. An extra, ventral tail (1) was also present. × 17.

The other survivor at this time was the one previously noted as having two tails. By the twenty-second day not only had these become distinct tails but also two other outgrowths from the bud had appeared (Fig. 28). Consequently, at this time there were present on the regenerating part the following structures: (1) A tail growing out from the mid-ventral line near the base of the new part. This bore a distinct collar region open on one side and bearing 6 cirri—a condition frequently found in tail-regeneration at mid-body levels. (2) A tail which had been terminal but which was now tipped ventrally. This tail had an anal collar and 9 cirri. The dorsal portion of the collar and the 4 cirri found there were shorter than these parts in the ventral region. This bud bore two pairs of notopodia near its base. (3) and (4) On the dorsal side of the mid-part of the terminal tail a newly-formed outgrowth was present as a result of which the tail was tipped

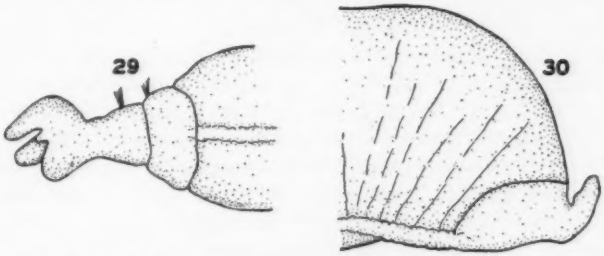


FIG. 29. Ventral view of a bud at twelfth segment after 17 days. It was probably a heteromorphic tail. × 13.

FIG. 30. Right lateral view to show bud formed at fourteenth segment after 33 days. Drawn with aid of camera lucida. × 22.

ventrally. This outgrowth consisted of two parts—a larger one lying in line with the longitudinal axis of the worm and terminating in a bilobed region with two notopodia both on the more dorsal lobe; a slender, slightly longer part extending to the right (of the original posterior piece of the worm) and ventrally. The dorsal blood vessel could be seen running forward and dividing to send a branch into each of two dorsal parts. In spite of all precautions this worm died during the following night.

Regenerates at *levels posterior to the eleventh segment* may be considered together. No data on the relative numbers of deaths and survivals at these levels are available. Unfortunately a number of experiments involving these were started without making any record of the original numbers of pieces. At the end of 10 days there were 115 survivals at these levels and in the experiments where records of

cutting were kept 92 had died. The actual total number of deaths was probably 8 to 10 times that number. Of the survivors, 59 still had bulbs of gut present and no new tissue evident and 55 had closed wounds but were without new tissue. One, regenerating at the twelfth segment, had a very short bud.

Twenty days after cutting there were 66 alive. Twenty-four of these had bulbs; 40 had the wound closed but no new tissue; 2 had small buds. One of the buds—at the twelfth segment—(Fig. 29) was probably a somewhat peculiar heteromorphic tail with 2 setigerous segments. This worm died on the twentieth day. The other regenerating piece (Fig. 30) had been cut at the fourteenth segment. After 33 days the new tissue present included a fairly large round mass over the wound surface from the anterior ventral part of which a slender portion extended forward and curved toward the dorsal side.

Discussion

In anterior, as in posterior, regeneration *Clymenella* tended to replace the segments lost. When six or less segments were removed they were exactly replaced. When 12 or more segments were removed, with 2 exceptions, no new bud was formed. In the intermediate region the percentages of survivors producing complete buds of approximately the usual shape were: at the seventh segment, practically 100; at the eighth, 75–85; at the ninth, 35–40; at the tenth, none out of 55 pieces; at the eleventh, none out of 14. At the last three levels many incomplete or irregular buds were formed. This is almost the exact reciprocal of the situation in posterior regeneration. In the latter no regeneration occurred in the anterior region, complete regeneration usually occurred in the posterior region, and in the intermediate region there was a progressive drop in the ability to regenerate as the cuts were made at more and more anterior levels.

It is interesting to compare regeneration in the laboratory with that under natural conditions. Of 1,364 worms examined during the summers of 1929–31, inclusive, only 33 showed evidence of having regenerated anteriorly. Of these, 12 had regenerated at the second segment, 10 at the third, 7 at the fourth, 2 at the fifth and 2 at the sixth. All of these had replaced exactly the parts lost. No worms were found which had replaced more than 6 segments. Since autotomy occurs only in the posterior half of the body, these regenerates must have resulted from accidental injuries. As such injuries could occur equally well in the middle and posterior regions of the body, it seems that under natural conditions such injured pieces are rarely, if ever, able to survive and to regenerate.

As in posterior regeneration, there was a fairly close parallel between the level of the cut and the ability to survive. In the region of the complete replacement of lost parts—at segments 1–6, inclusive—more than 85 per cent survived for 10 days or longer. In the region where buds were practically never formed—segments 12–21, inclusive—probably less than 15 per cent survived on the average, with only an occasional piece living after cuts at the most posterior levels. In the intermediate region there were the following percentages of survivals at 10 days: at segments 7, 8 and 9 about 70 each; at segment 10, 43; at segment 11, 26.

The response of *Clymenella* to the loss of an anterior portion of the body is different from that of most polychætes. We may mention a few cases, especially among genera having representatives in the Woods Hole region. According to Michel (1898) *Phyllodoce*, *Nephthys*, *Eulalia* and *Capitella* will not regenerate anteriorly. We have verified this for the first two. Czerski and Nusbaum (1905) kept capitellids for 25–27 days without the appearance of new buds. Czwiklitzer (1905) found that *Ophryotrocha* would produce new parts only when no more than portions of the first segment were lost. On the other hand, Berrill (1928) reported that *Chætopterus* would replace lost anterior segments up to and including the fourteenth. Posterior to that level anterior regeneration did not occur. This was an extension of the facts reported earlier by Gravier (1909). Pflugfelder (1929) found that *Diopatra* would rapidly replace anterior segments lost, apparently up to as many as fourteen.

In between these extremes are the cases where anterior regeneration occurs but only a few of the lost segments are replaced by newly-formed ones. In many cases the bodies of these worms are differentiated into regions, usually including a head, a thoracic region of a definite number of segments and an abdominal one of a variable number of segments. Vaney and Conte (1899) reported that *Spirographis* formed 2 new segments anteriorly after cuts in the anterior part of the body. The requisite number of thoracic segments was then obtained by metamorphosis of the abdominals. Watson (1906) found that *Potamilla* formed a head and one thoracic segment and then completed the thorax by metamorphosis. Berrill (1931) reported that *Sabella* produced a new head plus one or two collar segments and then completed the process by a metamorphosis of the necessary number of abdominal segments.

Clymenella does not fall exactly in any one of these three categories. It does tend toward a complete replacement of the lost part at anterior levels. In the mid-body region, in addition to many cases of irregular

buds, there were a number in which the usual arrangement of things occurred but not all of the lost segments were replaced. Perhaps one of the best examples of this is the worm which produced a peristomium and only 2 setigerous segments, all of good shape, after the loss of 10 (Fig. 18). This worm was kept 28 days yet it showed no evidence of a change in the nature of the body-wall in which there is ordinarily a very definite line of demarcation (at the ninth–tenth intersegmental region) between the distinctly thicker wall anteriorly and the very thin wall in the posterior part. As might be expected from this fact, no trace of a new fifth-segment collar appeared on the old twelfth segment. Also in none of these cases where buds deficient only in the number of segments were formed was there any indication of the addition of new segments at the posterior end of the body. Since this worm has a constant number of segments—22—there is no growing region at the posterior end. It might then be thought that there was not the right kind of cellular material for such additions, although such material does appear when a piece of the worm is amputated. The suggestion came to mind that, if a few segments were removed from the posterior end of a regenerate, deficient as the result of the formation of such an anterior bud, then the new posterior bud might contain more segments than the part cut off. Vigorous looking worms with anterior buds of this special type were not common. After these cuts for posterior regeneration were planned, only two regenerates developed which could be used for this purpose. One of these—after 21 days regeneration at the tenth segment—had produced 3 new segments to form a bud similar to the one mentioned above. The other piece had regenerated 4 new segments at the ninth after 26 days. Both were cut again at the eighteenth segment. The former died a little less than 2 days later and without the completion of wound closure. After 15 days the other produced a new bud consisting of 4 distinct segments. Since the full number of segments to be present in a bud in *Clymenella* is laid down at approximately one time, it does not seem probable that more would have been added to this bud if it had been kept longer. This evidence is, of course, too scanty for us to draw any conclusions from it. For instance, the result might possibly have been different if the second cut had been made 12–14 days or less after the worm was brought into the laboratory.

As in the case of posterior regeneration in *Clymenella*, it seems that these buds of good shape, but consisting of a small number of segments should be attributed, in part at least, to a scarcity of material. It should be borne in mind that the mid-region of the body is a rather specialized one. For example, it is in segments 7 to 10, inclusive, that the large nephridia of these worms are located.

In several cases, 2 of which are shown in Figs. 7 and 20, a pair of parapodia was present on the peristomium. In most species of polychætes, including *Clymenella torquata*, parapodia are not present on the first segment. This occurrence in certain buds might be interpreted as reversion toward what has been considered the primitive condition.

In anterior regeneration 11 cases of multiple or heteromorphic structures have been observed. In contrast with this, neither of these features was observed in posterior regeneration in *Clymenella* (Sayles, 1934). Two of these were duplications in the peristomium. Two others were head-buds of the early type with extra outgrowths similar to the early stages in the formation of multiple structures. Both of these died before any more definite developments. Two others were heteromorphic tails without extra structures. Three were weakly developed heads, each with a secondary tail-bud outgrowth. One was a heteromorphic tail bearing 2 extra parts, one a head, the other a tail. The remaining one of these was a heteromorphic tail with an extra tail and also two other rather large, extra parts which had no characteristic head or tail features. If we consider these structures from the point of view of the levels at which they developed, we find that 8 were at the tenth segment, 2 at the eleventh segment, and 1 at the twelfth. To understand the true frequency of these structures we may note that 7 of the 8 at the tenth segment were alive at 20 days; this represents 14 per cent of the 50 survivors which had produced new tissue. At this time the 2 at the eleventh segment were 17 per cent of the 12 new buds at this level. The one at the twelfth was the only bud produced at that level by any piece. In other words, this type of irregularity occurred with about the same frequency at the tenth and eleventh segments and in the only case of a bud differentiating posterior to the eleventh. In contrast to the situation in posterior regeneration in this worm, there was here a definite upset in the organization of the new material with the result that the new structure was frequently not of the same type as the one lost.

One further point of interest was the appearance of a bud showing spiral segmentation, a split segment and an intercalated half-segment. This is the only case of this type among several thousand regenerates of this worm which have been studied. Also no intact worm has been found which possessed such arrangements.

Ehlers (according to Buchanan, 1893, p. 541) suggested that intercalated segments were the result of regeneration of lost parts. Buchanan implies that Ehlers thought the irregularities to arise at the point of contact of old and new. Speaking for herself, she says,

"Even if the intercalation of half-segments were shown to be due to regeneration, it would still remain to be shown that the spiral arrangement is likewise due to it. The fact of spirals occurring in *Pentastomum*, where, as far as we know, regeneration does not take place, seems to me against this hypothesis." Morgan (1895), on the other hand, is of the opinion (p. 429) that "The large number of abnormal forms found in *Amphinome* is in part due no doubt to the frequent regeneration of portions of the body that takes place. The segments are broad, and for that reason it is surprising to find abnormal combination of the metameres so frequent." Unquestionably these features are very rare in *Clymenella*, in which the segments are extremely long. Also there can be no doubt about the fact that these peculiarities arose in regeneration. As far as the question of their appearance at the point of contact of old and new is concerned, the two spirals were adjacent to the old part. On the other hand, the half-segment and the split segment were at the distal part of the bud and separated from the spiral regions by a segment of apparently normal arrangement.

In connection with the origin of these peculiarities both Cori (1892) and Morgan (1892) thought that they were due to irregularities in the sizes and shapes of the developing mesodermal blocks. They attribute a spiral to a mesodermal block of one side, but not the other, being larger above than it is below, or vice versa. As a result, the single block of one side joins two of the other side. Then in another place this is offset to end the spiral. This bud in *Clymenella* is in agreement with such an explanation since there are the same number of segments on the right and left sides of the body, despite the fact that 4 irregular parts exist in a relatively short region.

Summary

1. Six or fewer anterior segments were exactly replaced.

2. Only 2 worms produced buds at cuts posterior to the eleventh segment. Most pieces cut at such levels died relatively soon.

3. In the region from the seventh to the eleventh segments there was an increase in deaths, a decrease in ability to produce complete buds, and a marked increase in the number of irregular buds.

4. At the ninth to eleventh segments a number of buds were of the usual type but possessed fewer segments than had been removed. Available evidence points to the conclusion that regenerates of this type do not undergo any metamorphosis of segments or any increase in number of segments at the posterior end of the body.

5. In several buds a pair of parapodia appeared on the peristomium which is ordinarily asetigerous.

6. Eleven cases of heteromorphic or double structures appeared, all confined to cuts at segments 10, 11 or 12. Two of these were double parts of the peristomium. Two were heads with extra structures possessing neither head nor tail characters. Two were heteromorphic tails without extra structures. Three were heads, each with a secondary tail outgrowth. Two were tails with extra outgrowths.

7. A most interesting bud appeared on one piece cut at the ninth segment. It possessed the following features: a segment split on the left side, a half-segment intercalated on the right side, and two spiral segments. One of the latter was a left-hand spiral of one and one-half turns, the other a right-hand spiral of possibly a little over one-half turn.

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APICAL DOMINANCE AND POLARITY IN MNEMIOPSIS LEIDYI, AGASSIZ[1]

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The locomotion of *Mnemiopsis leidyi*, as it moves along with its oral end forward near the surface of the water, serves to demonstrate that this animal possesses a definite polarity. The forward movement is a continuous reaction and is interrupted only when the animal bumps into some definite obstruction. Even this interruption is very brief, for immediately afterward the animal resumes its oral direction of movement. This locomotion of *Mnemiopsis* demonstrates a definite polarity within the rows of plates since the beating of the plates causes the animal to move, and this in turn indicates the presence of some sort of dominance. The proof of this dominance, with its location and character, has been reported by Child (1917, 1933) and Coonfield (1934). The conclusion reached thus far is to the effect that the apical organ, or region, exerts a dominance over the other parts of the body. In support of this conclusion, I offer further experimental evidence which is listed and discussed according to the following five headings: (1) Swimming-plate movement, (2) feeding reactions, (3) reversing parts of plate-rows by grafting, (4) reversing the mid-body section by grafting, (5) transplanting the apical organ.

Swimming-Plate Movement

The rows of swimming-plates of *Mnemiopsis* may be grouped into quadrants according to both their morphological and physiological features. Each quadrant contains two rows of plates, an adesophageal and an adtentactular (Figs. 3 and 4). These two rows may be considered as a unit which is coördinated with each of the other three similar units. This coördination is clearly shown by the synchronous movement of the plates as the animal progresses through the water. All plates stop beating instantly when the animal bumps into an object. Immediately after their cessation, either the swimming-plates reverse the direction of their effective beating for a very brief time, or the oral lobes contract. By either reaction, the animal is driven backwards for a short distance. In either effect, it seems clear that

[1] Contribution No. 13.

we can assume that some sort of centralized structure or region exerts a dominance and regulation over the locomotor organs. The view that this dominance is located in the apical end of the animal is supported by the origin and sequence of the regular movement of the plates. The plate at the apical end of each row contracts first, then is followed by each other plate in the row contracting in sequence. This successive beating produces a metachronal wave, according to Parker (1905). The direction of this wave is never reversed, even on an isolated part of the animal in which a reversal of the effective direction was induced (Coonfield, 1934).

Feeding Reactions

The feeding movements of *Mnemiopsis* were followed easily by observing specimens as they fed on clam juice containing a suspension of carmine. Every individual fed readily on this juice and the carmine particles made the tracing of the food passage, both on the outside of the body and within the body, quite convenient.

The clam juice was placed successively in very small quantities on all parts of the body surface, the entire group of reactions in response to each small quantity of food being recorded before more clam juice was placed on any other part. Positive feeding reactions followed only when the juice was placed on the adtentacular region, nearer the mouth than the apical end, on the lips, auricles, and lobes near the mouth (Fig. 3). The adtentacular region was chosen as the area on which to observe the feeding reactions principally because of its mechanical advantages. Therefore, the feeding reactions recorded here are those observed on the adtentacular region of the body.

As soon as the clam juice touched the adtentacular body surface, the swimming-plates of both rows nearby stopped their beating. This was due possibly to the intervention of the primary inhibitory action as described by Göthlin (1920). The very brief period of cessation was followed immediately by a reversal of the effective beating, which was also of very brief duration. Immediately after this reversal, the swimming-plates resumed their normal direction of beating toward the apical end of the animal. This reversal, though very brief in duration, was sufficient to initiate the moving of the food toward the mouth. While being moved in this direction, it spread out over the oval surface of the adtentacular region of the body (Figs. 3 and 4). In this region, the food was formed into a thin sheet by the addition of mucus from the body wall of the animal and was carried on toward the mouth and into the labial trough (Fig. 4) by the beating of very small cilia which cover this part of the body. Thence it was propelled

on to the mouth both by cilia and by the peristaltic contractions of the labial ridge (Fig. 4). The tentacles lying at the edge of the labial trough were caught in the food stream and were stretched out in the direction of the mouth. Some of them were even broken off, presumably by the current, and were taken into the mouth with the food. The above description of the feeding reactions of *Mnemiopsis* follows fairly closely that of Main (1928). He makes no mention, however, of the reversal of the swimming-plates, nor does he report the presence of cilia on the body surface of the animal. The lips were primarily responsible for pushing the food into the mouth. The food then passed on up the stomodeum (stomach) to the funnel (Fig. 3) by the effective beating of cilia which cover the entire lining of this region, and by the peristaltic contractions of the wall of the stomach. At the funnel, the food, in solution and in suspension, was propelled out into the paragastric, the tentacular, and the meridional canals by the beating of cilia which cover all the inner surfaces of these canals (Figs. 3, 4, and 5). The association of a meridional canal with the swimming-plates is shown in Fig. 5. Some food in the funnel was carried also up into the infundibulum (Fig. 1) by cilia, where it either returned to the lower funnel region and was carried out into the other canals or was discharged. Within one or two hours after an animal fed, it discharged a steady stream of materials through a pore in one angle of the infundibulum (Fig. 1). This pore, with its discharging reaction, has been reported by Main (1928). I am not certain, however, whether the pore is permanent or only temporary. All the animals that I observed discharging materials ejected them through only this one particular region. The pore is visible only when the actual discharge is taking place.

Reversing Parts of Plate-Rows by Grafting

The metachronal wave in each row of swimming-plates originates at the apical end and moves toward the oral end of *Mnemiopsis*, while the effective beat of these plates is toward the apical end. The origin and progression of the metachronal wave supports the view that the apical zone is responsible for the initiation of this wave. This zone, therefore, might well be considered as a region of dominance. Further support of this view is indicated by the synchronous rhythm exhibited by all plates of the body in their metachronism. That the rows of plates are polarized and are regions through which impulses pass, resulting in the movement of plates, has been reported by Child (1933), Coonfield (1934), and Parker (1905).

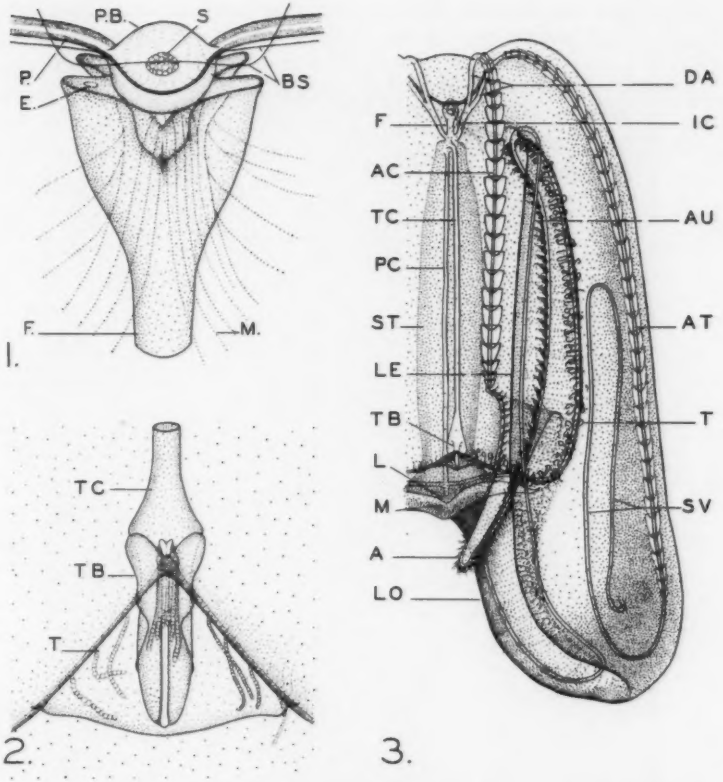
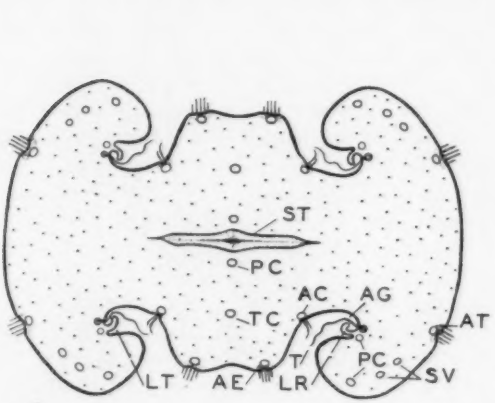


FIG. 1. A drawing of the infundibular funnel and polar body of *Mnemiopsis*. *BS*, body surface; *E*, excretory pore; *F*, infundibular funnel; *M*, muscles which terminate in the body wall; *P*, polar plate; *PB*, polar body; *S*, sense organ.

FIG. 2. A drawing of a tentacular bulb of *Mnemiopsis*. *T*, a lateral tentacle; *TB*, tentacular bulb; *TC*, tentacular canal.

FIG. 3. Drawing of a lateral view of *Mnemiopsis*. *A*, auricle; *AC*, adtentacular meridional canal with swimming-plates; *AT*, adesophageal meridional canal with swimming-plates; *AU*, auricular canal with plates; *DA*, adradial canals; *IC*, interradial canal; *F*, infundibular funnel with the polar body and sense organ above it; *L*, lip; *LE*, edge of lobe; *LO*, lobe; *M*, mouth; *PC*, paragastric canal; *ST*, stomodeum; *SV*, subventral meridional canal; *T*, lateral tentacle; *TB*, tentacular bulb; *TC*, tentacular canal.

Since the impulses which pass along the rows of plates are polarized, while these rows are in their natural position and orientation, the proof of the permanency of this polarity, which would test also the view of apical dominance, is revealed to some extent in the following experiments. A wedge-shaped piece of the mid-piece of either a single row or a similar section containing two adjacent rows, was cut out, rotated 180°, and grafted back in the same location. The adtentacular rows were cut and grafted because of certain mechanical advantages.



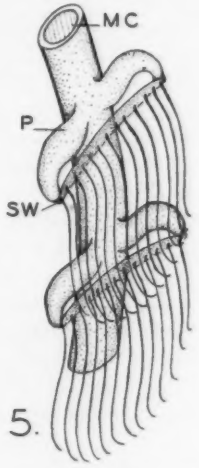


FIG. 4. A diagrammatic cross-section of *Mnemiopsis* in the region of the oral ends of the adtentacular rows. *AC*, auricular canal and plate; *AE*, adtentacular meridional canal and plate; *AG*, auricular groove; *AT*, adesophageal meridional canal and plate; *LR*, labial ridge; *LT*, labial trough; *PC*, paragastric canal; *ST*, stomodeum; *SV*, subventral meridional canals; *T*, lateral tentacle; *TC*, tentacular canal.

FIG. 5. A diagram of a meridional canal to show its association with the swimming-plates. *MC*, meridional canal; *P*, branch of the canal to the base of a plate; *SW*, swimming-plate.

The ends of the rows of the grafts were placed in contact with the ends of the intact rows. In this new location, the rows of the grafts were exactly opposite, morphologically and according to their polarity, to these characteristics of the intact rows. The grafts were held in place by strands of cotton for about two hours to permit sufficient healing. During the healing period, the movement of the plates within the grafts was not in harmony with the synchronism of the other plates of the body, though these were perfectly synchronized.

The plates, however, in a single graft were synchronized with each other and the direction of both their metachronal and effective beating was the same as before rotation. In grafts involving a single row of

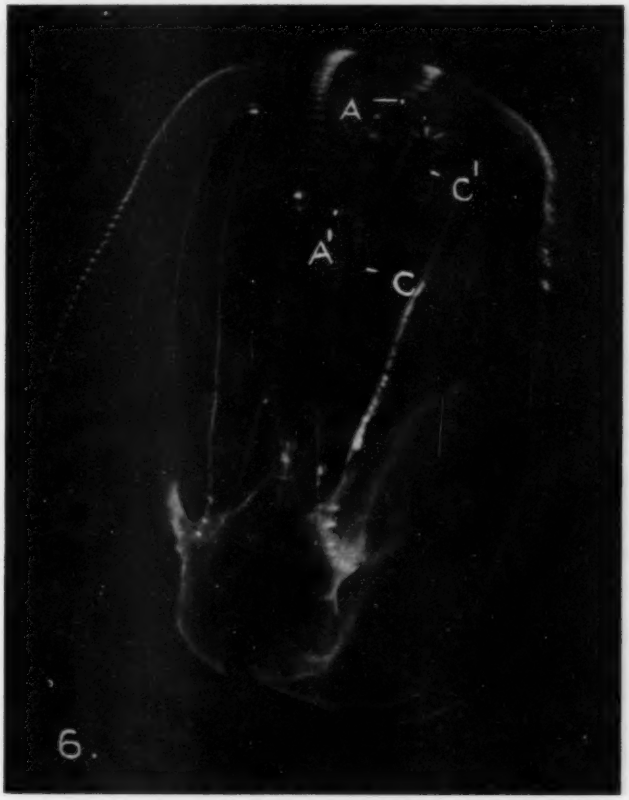


FIG. 6. This is a photograph of a living animal showing the result of rotating a piece of an adtentacular row. *A*, the point where the row was cut near the apical region; *A*', the end of the graft which was joined to the intact row at *A*. The row between *A* and *A*' shows the regenerated row. *C*, the point where the row was cut near the oral region; *C*', the end of the graft which was joined to the intact row at *C*. The row between *C* and *C*' shows the regenerated row. The row between *A*' and *C*' represents the rotated graft.

plates, this condition existed until the effects of healing and regeneration gave the following result. Within thirty hours after the grafts were made, a canal, regenerating from the end of each of the intact

oral part and apical part, made connection with the corresponding morphological end of the graft (Fig. 6). At this time, the beating of the plates of the graft became synchronized with that of the other plates. The rotated grafts containing sections of two adjacent rows of plates responded in the following manner. The tips of the rows at each end of the graft moved in toward each other. The ends of the intact rows at the graft region also moved toward each other. The rows of plates remained in this state, neither fusing with each other nor regenerating, as in the case of the experiments involving only one row. Here again the metachronism and the effective beat of the plates in the graft continued as had been established before the

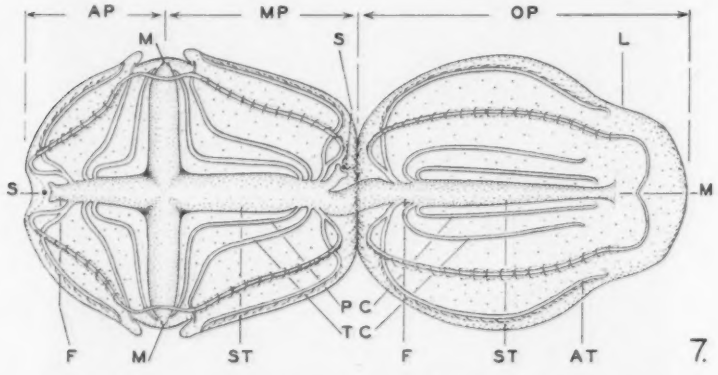


FIG. 7. A drawing of a living animal which had its mid-section rotated and grafted back in place. *AP*, apical piece; *AT*, adtentacular canal and plates; *F*, infundibular funnel; *L*, lobe; *M*, mouth; *MP*, mid-piece; *OP*, oral piece; *PC*, paragastric canal; *S*, sense organ; *ST*, stomodeum; *TC*, tentacular canal.

rotation was made. The total actions exhibited by these grafts in the matter of independence of plate movement were the same as those of a single, isolated piece, as reported by Coonfield (1934), who stated that the plates of an isolated piece beat as a coördinated unit.

REVERSING THE MID-BODY SECTION BY GRAFTING

In order to test further the problem of apical dominance and the established polarity of impulses within the rows of swimming-plates, as set forth in the preceding section of this report, the following experiments were executed. The body of *Mnemiopsis* was cut across twice between the apical and oral ends, thus dividing the body into

three parts, an apical, a middle, and an oral one (Fig. 7). The mid-section was rotated 180° and then grafted in place between the apical and oral sections. During healing and fusion, the three pieces were held together by strands of cotton and strips of filter paper. The mid-piece contained only the parts of the rows of plates and the digestive tract that are naturally in this region (Fig. 3). The apical piece contained the apical organ, the funnel and infundibulum with the interradial canals, and the bases of the meridional canals under the rows of plates. The oral piece contained the mouth, the tentacular bulbs (Fig. 2), the lobes, the auricles, and the ends of the meridional canals in the plate rows. Due to the severity of the operation and the mechanical handicaps accompanying it, only a few of the experimental animals lived. In most cases the oral piece of these operatees failed to fuse, leaving only the apical and the mid-piece healed together. Both the two-piece grafts and the three-piece grafts were retained and observed.

The results obtained from those with the three parts healed together are shown in Fig. 7. The mid-piece regenerated an apical organ and a funnel with its canal connections. The stomodeum of the middle piece fused with that of the apical piece. At the region of the fusion, digestive tubes, with their mouths and lips, were regenerated. The corresponding meridional canals of the two pieces joined. The oral piece failed to regenerate an apical organ, but an infundibulum with its canal connections was formed. This structure fused with the infundibulum of the mid-piece (Fig. 7). Food, taken in at the mouth of the oral piece, passed up its stomodeum and into the same structure of the mid-piece. Also, in these experimental animals, the swimming-plates of each piece behaved as though the piece constituted an individual organism. Thus the metachronal and the effective beating directions in the plates of the mid-piece in its rotated position were opposite to those of the apical and oral pieces. In all experiments wherein only the apical and the middle pieces joined, the mid-piece regenerated an apical organ and an infundibulum with its canal connections. The corresponding meridional canals and stomodeum of each failed to fuse. These operatees subsequently either separated or died.

Transplanting the Apical Organ

An apical organ (Fig. 1) of one animal was grafted to the body of another animal, about midway between its apical and oral ends and on the adtentacular surface. This transplant contained some of the

funnel and the bases of the interradial canals. The grafts healed in very quickly, and within two days the regenerating ends of their interradial canals joined the adradial canals of the host. These new canals developed new plates which, according to the directions of their metachronal and effective waves, were coördinated by the transplant. Some of these plates, due to their relationship to those of the host and to their regulation by the transplant, beat in a direction opposite to that of the respective rows of the host. Within ten days after the transplant had healed in the host, it had been completely absorbed. This absorption was a continuous one, lasting about four days, during which time the transplant became less vigorous on each succeeding day.

Discussion

Any region of an organism which exerts a regulating influence over body activities, such as growth, regeneration, and the transmission of impulses or substances to other parts of the body, is regarded as possessing physiological dominance. This physiological dominance, according to Child (1928), may possess a correlative factor which undergoes a decrement in intensity with an increase of distance from the point of origin. Then a region of *Mnemiopsis* if it exerts a regulating influence over the other parts of the body possesses a physiological dominance. Such a region, the apical one, is located in a depression at the aboral end of the animal. It contains a polar body (apical organ) which lies just above the tip of the infundibulum. This polar body (Fig. 1) is made up of the following visible parts. 1. A concentration of lithic materials in the shape of a morula enclosed within a bell-shaped capsule. This capsule is composed of fused cilia (Mayer, 1912) and is supported upon four ciliated grooves which connect each with an interradial canal. 2. A polar plate which lies on the surface of the depression, surrounds the capsule, and extends to either side of the capsule in the stomodeal plane. 3. A concentration of nervous elements which radiate out into the plate rows. These elements are thought to be responsible for the coördination of the plate movements.

The swimming-plates of the eight rows of *Mnemiopsis* are synchronized as a unit in their beating while the animal is in its natural condition. This synchronism of the eight rows is rather significant, since, according to Coonfield (1934), each row shows the highest type of unity in the animal. That these units are regularly brought under the control of a particular region, presumably the apical organ, is convincing in demonstrating that this organ is a region of dominance.

The feeding reactions show the presence of a secondary polarity which is subjective to the primary one. This secondary reaction is temporarily dominant during feeding, requiring the presence of food as a stimulus. That this secondary polarity is not permanent is shown by the temporary reversal of the swimming-plates in the region stimulated. This reversal is rarely executed except during feeding. Just after the food is taken into the mouth, the primary polarity becomes dominant again and supercedes the secondary one.

The permanency of the polarity of the rows of plates, which I believe supports the view of apical dominance, is shown very definitely by the experiments in which the mid-section of a row was rotated and grafted back in place. The polarity of the rotated section of row had been so well established previous to the operation that it was maintained throughout the healing and regenerating period. This is significant since in its new location this polarity was just opposite the primary polarity of the body. The permanency of this polarity was further demonstrated by the directed regeneration of the ends of the apical and the oral parts of the row. Here the end of the apical part was in contact with the oral end of the graft, but instead of fusing with this physiologically opposite end, the apical part regenerated a canal which avoided the graft until it reached the end with which it had been originally in contact. The end of the oral part was in contact with the apical end of the graft, but instead of fusing with it, the oral part regenerated a canal which also avoided the graft until it reached the end with which it had been in contact. The experiments involving parts of two adjacent rows did not show any regeneration. It is probable that the size of the rotated piece mechanically prevented regeneration of rows. Despite the fact that regeneration did not take place, the ends of the rows of the rotated piece did not fuse with those of the apical or oral parts.

The experiments involving the rotation of the mid-section of the body of *Mnemiopsis* confirm the interpretations of the experiments cited in the preceding paragraph and show further that the apical organ exerts a dominance over the other parts of this animal. This additional evidence is brought out by the regeneration of an apical organ at the aboral end of the rotated piece. Apical polarity had been so firmly established in the body of the animal before the operation that the apical end of the graft formed an organ even though it was joined to the part of the body having an apical organ. I believe that during the period between the cutting of the animal and the actual fusion of the middle and the apical pieces the correlative factor had

established a dominance in the apical end of the mid-piece. This dominance promoted the regeneration of an apical organ. Further indication of this correlative factor lies in the fact that the oral piece failed to form this organ. The oral piece fused with the mid-piece and its regenerated infundibulum joined that of the mid-piece.

The fact that the transplant in the experiments involving the transplantation of an apical organ behaved at first as an independent unit is significant. During the first few days after the operation, it maintained this unity. However, after four days, this unity of action was less marked. This decrease in independence was carried to completion within ten days, at which time absorption was complete.

This view of apical dominance is supported by Child (1917), who found that in response to KCN treatment the cessation of rhythmic plate-movement of *Mnemiopsis* occurred first at the aboral end of a row. He reports further that, according to the relation between susceptibility and general metabolic condition, the aboral end of a row is the region of highest metabolic rate in this gradient, and from this point, the rate decreases in the oral direction. Hanström (1928), in discussing the subepithelial nervous tissues of cœlenterates, believes that the diffuse plexus is transformed into a longitudinal axis, and in the ctenophores, this axis is connected with the apical organ. The opinions of these two investigators are confirmed by the experiments cited and discussed in this report.

Conclusions

1. Apical dominance in *Mnemiopsis* is indicated by each of the following: *a*. The plates of all eight rows are synchronous in their beating. *b*. Following temporary reversal of the effective wave during feeding, the original direction of this movement becomes reëstablished. *c*. Apical polarity within a single row persists even though a mid-section of the row is rotated by grafting. *d*. A rotated mid-piece retains its original polarity and regenerates an apical organ. *e*. A transplanted apical organ retains its identity at first, but eventually becomes absorbed.

2. The intensity of dominance in the normal animal decreases as the distance from the apical region increases.

3. Apical dominance and its resultant effects account for the various activities and environmental responses of the organism.

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DECOMPOSITION OF ORGANIC MATTER IN SEA WATER BY BACTERIA

III. Factors Influencing the Rate of Decomposition [1]

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The question has been raised by Krogh (1934*a*, *b*) and other investigators, whether or not the organic matter in sea water can be utilized by bacteria and can be broken down with the liberation of the constituent elements in mineralized forms. It has been previously reported (Waksman and Carey, 1935*a* and *b*) that the organic matter in sea water is not in a resistant state, but can be readily attacked by the bacteria found in natural water, when this water is placed under favorable conditions. These investigations have further brought out the fact that this organic matter is sufficient to support an active bacterial population. The rapidity of decomposition of the organic matter by bacteria is most conveniently determined by the rate of oxygen consumption in the water and by the liberation of the nitrogen in an available form; the latter process can best be measured in an indirect manner, namely by the decomposition of a non-nitrogenous organic substance, such as glucose, added to the water, since the process of glucose decomposition was found to be controlled by the concentration of available nitrogen or by the rate of its liberation. It was thus calculated that about fifty per cent of the total organic matter in the water is readily attacked by the bacteria, under favorable conditions; of this decomposed organic matter, about sixty per cent is completely oxidized and about forty per cent converted into bacterial cell substance.

Among the factors influencing the rate and extent of decomposition of the organic matter in the water, the following were found to be of paramount importance: 1. The temperature of the water; the lower the temperature, the smaller is the amount of organic matter decomposed in a given period of time or the longer does it take to bring about the same degree of decomposition. When water taken in the neighborhood of Woods Hole was placed at definite temperatures, in

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closed containers, the amount of oxygen consumed in 6 days, per liter of water, was 0.26 cc. at 2° C., 0.83 cc. at 12°, 1.39 cc. at 22° and 1.14 cc. at 34° C. 2. Abundance of available organic matter. This was demonstrated by the fact that when water was filtered through different types of filters, which tended to remove some of the suspended organic matter, such as Seitz filters, the rate of decomposition of the organic matter, as measured by the amount of oxygen consumed in a given period of time, was considerably reduced. 3. The water mass itself, as modified by distance from bottom, by its relation to photosynthetic zone, by latitude, by movements, especially oceanic circulation and vertical mixing. In water taken from the Gulf of Maine, the greatest oxygen consumption took place at a depth of 40 meters,

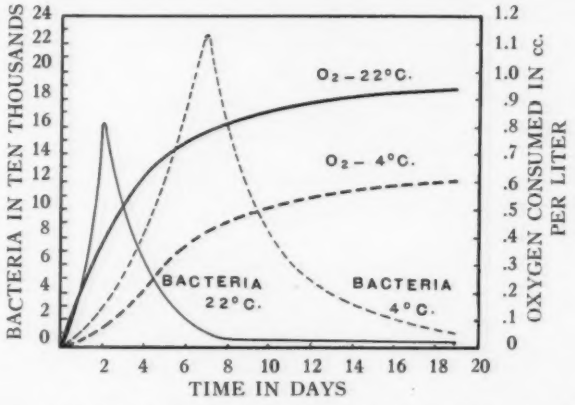

FIG. 1. Influence of temperature upon bacterial development and oxygen consumption in sea water.

while at Georges Bank, with greater vertical circulation, in the water just above the bottom. 4. Concentration of available inorganic nutrients; the influence of available nitrogen upon the decomposition of carbohydrates has already been mentioned; certain preliminary investigations also point to the significance, in this process, of available phosphorus.

Influence of Temperature

In order to illustrate further the effect of temperature upon the course of bacterial development and oxygen consumption, the results of a detailed and controlled experiment are reported in Fig. 1. The oxygen bottles were incubated under water. Although the maximum rate of bacterial development was reached as 22° C., within 2 days,

and at 4° C., within 7 days, the consumption of oxygen continued even after that maximum was attained. The ratio between the periods of maximum bacterial development at the two temperatures was about 4 : 1. It is interesting to note that exactly the same amount of oxygen was consumed at both temperatures when this maximum was attained, namely 0.45 cc. per liter at 22° C. and 0.47 cc. at 4° C.

Influence of Organic Compounds

The second experiment illustrates the effect of two different forms of nitrogen upon the decomposition of a non-nitrogenous organic substance (glucose) added to sea water (Table I). In all cases, the

TABLE I

Influence of nitrate and ammonia nitrogen upon the decomposition of glucose in sea water *

Glucose added	Source of nitrogen	Amount of nitrogen added	Bacteria in 1 cc.		Oxygen consumed	
			3 days	7 days	3 days	7 days
mgm. per liter		*mgm. per liter*			*cc. per liter*	*cc. per liter*
0	0	0	360,000	25,500	0.54	0.73
0	$NaNO_3$	0.1	345,000	17,000	0.52	0.69
0	$(NH_4)_2SO_4$	0.1	540,000	15,500	0.60	0.91
1.5	0	0	515,000	54,000	0.65	1.32
3.0	0	0	384,000	44,000	0.72	1.54
6.0	0	0	340,000	52,000	0.64	1.54
1.5	$NaNO_3$	0.1	1,930,000	42,500	0.95	1.27
3.0	$NaNO_3$	0.1	1,650,000	7,000	1.79	2.43
6.0	$NaNO_3$	0.2	3,200,000	7,200	2.74	3.69
1.5	$(NH_4)_2SO_4$	0.1	1,750,000	7,800	1.27	2.10
3.0	$(NH_4)_2SO_4$	0.1	1,880,000	6,000	1.76	2.37
6.0	$(NH_4)_2SO_4$	0.2	4,600,000	24,000	2.72	3.77

* Fresh water contained 1,050 bacteria in 1 cc. and 5.43 cc. oxygen per liter; bottles incubated at room temperature.

water received 2 mgm. K_2HPO_4 per liter, to eliminate any effect due to a lack of available phosphorus in the water. The initial water contained 1,050 bacteria in 1 cc. and 5.43 cc. of oxygen per liter; the water was incubated at 22° C. In the decomposition of glucose added to sea water, the available nitrogen is definitely shown to be an important controlling factor; in the absence of available nitrogen, the addition of glucose did not result in any appreciable increase in oxygen consumption and bacterial development within three days, namely the time of most active bacterial multiplication in stored sea water; at a later period, however, active glucose decomposition sets in. However, there was still insufficient nitrogen available from the

decomposition of the organic matter in the water, even after 7 days, to result in the oxidation of appreciable quantities of glucose; when 1.5 mgm. glucose was added per liter, the increase in oxygen consumption was 0.59 cc.; with another 1.5 mgm. glucose, the increase was only 0.22 cc., while the third portion of 1.5 mgm. glucose brought no further increase at all. It is interesting to note that the oxidation of glucose in 7 days resulted in an increase in oxygen consumption just about equivalent to that which took place in the water itself. The addition of 0.1 or 0.2 mgm. nitrogen to the water receiving the glucose greatly increased the rate of its decomposition. The two

TABLE II

Oxygen consumption in the decomposition of amino acids in sea water by bacteria

Compound	Amount added	Oxygen consumed, cc. per liter			Theoretical oxygen required for complete oxidation of 1 mgm. of compound *	Bacteria in 1 cc.
		2 days	5 days	Per 1 mgm. of amino compound used		2 days
	mgm. per liter					
Water alone	—	0.71	1.01	—	—	820,000
Glycocoll	2	0.65	1.19	—	—	1,100,000
Glycocoll	4	1.42	1.95	0.38	0.64	2,050,000
Alanine	2	1.39	2.03	—	—	1,600,000
Alanine	4	2.03	2.94	0.46	1.08	3,700,000
α-Phenylalanine	2	2.08	2.77	—	—	950,000
α-Phenylalanine	4	3.66	4.95	1.09	1.93	1,200,000
Glutamic acid	2	1.31	1.78	—	—	1,950,000
Glutamic acid	4	2.30	3.24	0.73	0.98	3,550,000
Tyrosine	2	1.99	2.59	—	—	1,090,000
Tyrosine	4	3.57	4.58	1.00	1.68	1,600,000
Asparagine	2	0.82	1.23	—	—	1,120,000
Asparagine	4	1.32	1.97	0.37	0.73	2,100,000

* For complete oxidation of compound, assuming that nitrogen is left as ammonia.

sources of nitrogen had about the same effect upon the rapidity of utilization of glucose by bacteria.

The consumption of oxygen takes place not only at the expense of the carbohydrates in the water, but also in the decomposition and oxidation of the organic nitrogenous constituents, as brought out in Table II. Four amino acids, namely glycocoll, *d*-alanine, α-phenylalanine, *d*-glutamic acid and tyrosine, and asparagine, were added to fresh sea water, in concentrations of two and four milligrams per liter. Consumption of oxygen and bacterial multiplication were determined, after two and five days incubation of the water, in the dark at 20° C.

For the calculation of the amount of oxygen consumed per unit of nitrogenous compound, the results obtained after five days incubation were used. The increase in oxidation for the addition of 4 milligrams of compound above the water receiving the 2-milligram portions was used as basis of interpretation of the results. This experiment brought out the fact that various simple nitrogenous compounds are readily oxidized by marine bacteria in sea water; a half or more of the theoretical amount of the different compounds was destroyed in 5 days, as measured by the amount of oxygen consumed; the rate of oxidation of the various compounds was found to vary with their specific chemical nature. These results tend to explain the rapid rate of decomposition of zoöplankton in sea water, as compared with that of the marine algæ (Waksman, Carey and Reuszer, 1933). Because of the greater proportion of available nitrogen in the former, their

TABLE III

Influence of light upon bacterial development

Incubation	Oxygen, cc. per liter		Bacteria in 1 cc.	
Days	Dark	Light	Dark	Light
0	5.60	5.60	3,580	3,580
2	5.40	5.91	10,500	130,000
4	5.10	5.69	2,500	11,000
6	5.17	6.50	2,200	8,500
9	4.86	6.69	6,500	9,600
12	4.72	6.97	15,000	14,200

decomposition is accompanied by the liberation of nitrogen in the form of ammonia or as other simple compounds, while the decomposition of the second is accompanied by nitrogen consumption. The importance of the carbon-nitrogen ratio of the organic complex is evident. Substances with a narrow ratio will, on decomposition, result in a rapid liberation of the nitrogen. This nitrogen is partly utilized by the bacteria in the process of decomposition of the carbohydrates and is partly left in the water in a form available for the growth of the algal and diatom populations.

Relationship of Photosynthesis and Phytoplankton Development to Bacterial Activities in Sea Water

A parallel development of diatoms and bacteria in sea water has previously been reported (Gran, 1933, and Waksman et al, 1933). It is quite likely that this is due primarily to the fact that the bacteria

TABLE IV

Rapidity of bacterial decomposition of freshly synthesized plant substances

Incubation	Oxygen, cc. per liter			Bacteria in 1 cc.		
Days	Dark	Light	Light followed by dark for 5 days	Dark	Light	Light followed by dark for 5 days
0	5.41	5.41	—	10,000	10,000	—
3	4.95	5.67	5.04	28,000	17,000	14,500
6	4.79	5.52	?	9,000	19,500	?
10	4.55	5.84	5.43	7,100	6,000	9,500
20	4.47	6.96	5.91	20,500	13,000	18,300
30	4.30	7.60	—	17,500	9,000	—

feed either upon the organic substances secreted by the diatoms or upon those cells which, because of mechanical disintegration, become subject to bacterial attack. Fresh sea water was placed in oxygen bottles; some of these were incubated in the darkness at room temperature and under water, and some were exposed to the light, also under water. At definite intervals, some of the bottles were removed

TABLE V

Synthesis and decomposition of organic matter in sea water

Incubation, days		Oxygen, cc. per liter			Bacteria in 1 cc.		
Harbor	Laboratory	Dark	Light	Light followed by dark	Dark	Light	Light followed by dark
0*	—	5.80	5.80	—	—	—	—
7	7	4.60	6.19	5.01	—	—	—
7	14	—	—	4.05	—	—	—
0†	—	5.37	5.37	—	—	—	—
7	9	4.59	6.11	5.01	2,000	14,630	46,900
7	18	—	—	4.11	—	—	17,250

* Experiment begun on July 27.
† Experiment begun on August 10.

and analyzed for oxygen and bacterial numbers (Table III). The results show that continued decomposition of the organic matter took place in the darkness, while, in the light, organic synthesis predominated, as shown by the liberation of oxygen; the newly synthesized material supported a more abundant bacterial population.

In order to demonstrate to what extent this freshly synthesized organic matter can be decomposed by the bacteria in the water, the above experiment was repeated. Some of the bottles, after exposure to light for varying periods of time, were incubated, for a period of 5 days, in the darkness (Table IV). The synthesized organic substances are found to be readily subject to bacterial decomposition, as shown by the rapid reduction in the oxygen, when photosynthesis is excluded.

The results of two other experiments are reported in Table V. Sea water was placed in light and dark bottles and incubated directly

TABLE VI

Decomposition of organic matter in water from Gulf of Maine and Georges Bank, in the spring *

Station number	Depth	Temperature of water	Bacteria in 1 cc. of water	Water incubated for 5 days at room temperature			
				Water alone		Water and glucose †	
				Oxygen consumed	Bacteria in 1 cc.	Oxygen consumed	Bacteria in 1 cc.
	meters	*° C.*		*cc. per liter*		*cc. per liter*	
2441	5	7.5	—	1.28	621,000	3.68	1,375,000
2441	50	4.3	—	0.79	77,500	1.44	99,000
2441	225	5.8	—	0.71	24,000	1.30	—
2442	5	7.0	55	0.88	200,000	1.08	164,000
2442	50	3.9	8.5	0.66	48,300	1.46	714,000
2442	225	5.6	1	0.48	79,500	0.57	79,000
2443	5	6.9	2	1.00	27,400	1.69	71,000
2443	25	7.9	7	0.77	36,000	1.11	36,000
2444	5	6.35	19	0.98	96,000	1.13	101,500
2444	33	6.44	8	0.52	18,000	0.97	85,500

* Samples taken on May 20, 1935.
† Two mgm. per liter.

in the harbor, about five feet below the surface; a modified procedure of Winberg (1934) was employed. Here as well, some of the bottles, which have been exposed to light in the harbor for a period of 7 days were later incubated in the darkness, for varying periods of time. The temperature of the outside water was about 15° C. and of the tank in laboratory, about 20° C. The newly synthesized organic matter was again found to be rapidly decomposed, as shown by the disappearance of the oxygen and by the increase in bacterial multiplication, when photosynthesis was stopped.

Decomposition of Organic Matter in Sea Water at Different Seasons of Year and at Different Latitudes

An attempt can now be made to interpret the results obtained on the decomposition of the organic matter in sea water taken from a group of stations, at different depths, and at different seasons of the year. Water taken in the spring of the year from both deep (Gulf of Maine) and shallow (Georges Bank) stations showed the largest amount of rapidly decomposing organic matter at the surface, gradually decreasing with depth (Table VI). The amount of oxygen consumed in the presence of glucose was, on the average, just about

TABLE VII

Bacterial activities in the water of the Gulf of Maine in summer and fall

Date of sampling of water	Depth	Water incubated 5 days at room temperature	
		Oxygen consumed	Bacteria in 1 cc.
	meters	*cc. per liter*	
August 5, 1934	5	0.86	—
August 5, 1934	40	1.26	—
August 5, 1934	229	1.45	—
November 22, 1934	5	0.43	34,000
November 22, 1934	40	0.90	102,500
November 22, 1934	236	0.70	244,000
November 22, 1934	5	0.64	130,000
November 22, 1934	40	0.72	140,500
November 22, 1934	213	0.59	211,500
July 8, 1935 *	0	0.42	225,000
July 8, 1935	50	1.02	182,300
July 8, 1935	125	0.67	214,000
July 8, 1935	0	0.13	373,000
July 8, 1935	50	0.77	339,000
July 8, 1935	200	0.35	191,000

* Incubation of water, 3 days.

twice as high as in the water alone; this would tend to confirm the previous conclusions concerning the liberation of an equivalent amount of nitrogen in an available form, in the decomposition of organic matter in sea water. Water taken from the Gulf of Maine stations, in the late summer and fall, showed an increase in readily decomposable organic matter with increasing depth, then decreasing again in the water nearer to the bottom; the same was true also of the water taken in the Gulf of Maine stations during the following summer (Table VII).

Several stations were taken, during the winter of 1935, from Cape Cod Light to Bermuda; the rate of oxygen consumption and

bacterial multiplication in the water from those stations, kept in closed bottles at a given temperature (18–22° C.) and for a certain period of time (5 days), was determined. The water was filtered through sterile sand filters, so as to eliminate the interference of coarser material. The amount of oxygen consumed was reduced appreciably by the filtration process, namely, in one test from 0.70 to 0.35 cc. per liter and in another from 1.26 to 0.74; this was due to the fact that the readily decomposable larger diatoms, protozoans and other forms of life were removed. The most striking result of these experiments (Table VIII) is the poverty of the waters close to

TABLE VIII

Oxygen consumption in water taken from a series of stations from Cape Cod to Bermuda

Station number	Position	Depth	Temperature	Oxygen consumed*	Bacteria in 1 cc.	
					Start	5 days
		meters	*° C.*	*cc. per liter*		
2308	42° 21′ × 69° 34′	5	5.2	0.74	414	550,000
2309	42° 32′ × 69° 13′	5	5.2	1.00	614	213,750
2310	41° 27′ × 69° 33′	5	4.3	0.92	83	65,800
2311	41° 18′ × 69° 21′	5	4.5	0.96	68	132,000
2312	40° 12′ × 68° 21′	5	9.4	—	141	215,000
2312	40° 12′ × 68° 21′	50	5.4	0.83	399	207,500
2312	40° 12′ × 68° 21′	190	3.4	0.95	3,000	375,000
2313	38° 25′ × 68° 18′	5	11.8	0.63	52	169,000
2314	38° 20′ × 68° 17′	5	20.0	0.78	62	347,000
2315	37° 42′ × 68° 02′	5	21.5	0.62	412	204,000
2316	36° 09′ × 67° 30′	5	18.5	0.75	52	78,200
2317	33° 56′ × 65° 52′	5	19.1	0.10	66	79,000
Grog's Harbor, Bermuda		1–6	17.0	0.24	78	16,500

* Five days incubation at room temperature.

Bermuda. Although sufficient bacteria were found in the water and especially in the bottom material (174,000 bacteria in 1 gram of fresh material), there was not sufficient organic matter to support active bacterial development.

Influence of Oxygen Tension

The concentration of oxygen in the water was previously found (Waksman and Carey, 1935*a*) to have an important influence upon bacterial activities and upon oxygen consumption, when the water was allowed to incubate. Those results were obtained by the use of Seitz-filtered water. It was found in subsequent studies that this filtration

process removes a large part of the suspended and readily decomposable organic matter in the water, thus modifying considerably the extent of decomposition of the organic matter. This is illustrated in Table IX. In the unfiltered water, the organic matter decomposed readily even at the lowest concentration of oxygen; in the Seitz-filtered water, however, the amount of organic matter decomposed with a low oxygen content was much less. With an increase in the concentration of oxygen in the water, the rate of organic matter decomposition, although remaining less in the filtered water, approaches that of the unfiltered water. These results tend to establish certain facts: (*a*) the small amount of organic matter removed by filtration through Seitz filters undergoes rapid decomposition; (*b*) the oxygen tension required for the decomposition of the organic matter passing through the filter is greater; (*c*) the type of the bacteria developing on the organic

TABLE IX

Influence of oxygen tension in unfiltered and Seitz-filtered sea water upon bacterial activities

Treatment of water	Unfiltered water				Seitz-filtered water			
Oxygen content at start, cc. per liter	0.57	2.66	4.97	5.28	1.14	2.38	2.89	4.94
Oxygen content, after 8 days	0.05	2.16	4.24	4.61	0.89	2.07	2.50	4.40
Oxygen consumed	0.52	0.50	0.73	0.67	0.25	0.31	0.39	0.54
Bacteria in 1 cc., after 8 days	11,500	7,000	2,150	1,300	9,000	230,000	122,000	102,000

matter in the Seitz-filtered water is different, as shown by the fact that much larger numbers are produced accompanied by a much smaller amount of oxygen consumed. The difference in the numbers of bacteria given by filtered and unfiltered water may point to radical differences in the chemical nature of the two fractions of the organic matter in the water. This confirms some observations previously reported (Waksman and Carey, 1935*a*).

Continued Decomposition of Organic Matter in Stored Water

In all previous experiments, only short incubation periods were employed. The question naturally arises as to what happens when prolonged periods of incubation are used. In order to determine this effect, three large carboys were carefully washed and filled with sea water as follows: (*a*) fresh water from Woods Hole Harbor, (*b*) same water filtered through a column of washed sterile sand, (*c*) fresh

water from Gulf of Maine. Once a week, 1,200-cc. portions of water were removed and placed in oxygen bottles; the amount of oxygen absorbed in 7 days, by incubating the water in the dark, was then determined. The carboys were well shaken every day so as to increase the oxygen content of the water. The results (Fig. 2) show that the unfiltered harbor water gave the greatest amount of decomposition and that the rate of decomposition of the organic matter in the water was gradually reduced. The total amount decomposed in 6 weeks per liter of water, in terms of oxygen consumed, was 1.64 for the unfiltered harbor water, 1.19 for the filtered harbor water and 1.24 for the Gulf of Maine water.

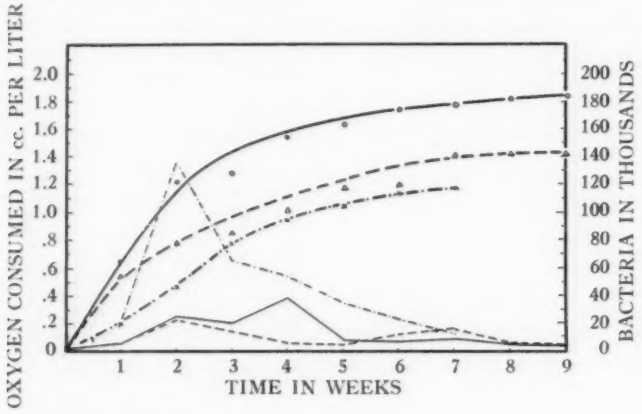

FIG. 2. Changes in bacterial numbers and oxygen consumption upon continued decomposition of organic matter in sea water. Heavy lines represent oxygen consumption; light lines, bacterial numbers. Heavy unbroken line, curve for unfiltered water from Woods Hole; heavy broken line, curve for filtered water from Woods Hole; ·—· represents curve for unfiltered water from Gulf of Maine.

SUMMARY

1. Investigations are reported on the decomposition of organic matter in sea water placed under controlled laboratory conditions. It has been found that, under these conditions, the organic matter in the water, both in suspension and in solution, can undergo rapid decomposition. It is quite possible that a change in temperature, resulting from the sudden warming up of the water, may bring about not only an increase in the biological reactions, but also a change in their very nature; this limitation need not invalidate the results obtained, since these results are not a measure of what actually

happens in nature under different conditions, but what may happen under any one set of conditions.

2. The abundance of readily decomposable organic matter in sea water was measured by the rate of oxygen absorption in the water, incubated under uniform conditions. The rapidity of liberation of nitrogen in the decomposition of the organic matter can be measured by the rate of decomposition of glucose added to the water.

3. The amount of organic matter in sea water readily attacked by bacteria varies with the rate of production of the organic matter, with photosynthesis and with the water mass; at the surface of the water, it falls off as one goes southward.

The authors are indebted to Mr. T. C. Cordon, for assistance in making the oxygen determinations.

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THE KINETOCHORE OR SPINDLE FIBRE LOCUS IN AMPHIUMA TRIDACTYLUM

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In another place I have expressed the conviction that the mitotic movement of chromosomes is closely associated or perhaps even dependent on the activities of the kinetochore (Schrader, 1935). In taking such a position I of course make no claim to originality, for the belief that the kinetochore is of the utmost importance in mitosis has become rather general in recent years, even though it is one that is more often tacitly assumed than openly stated. Indeed Metzner (1894), the first investigator to describe these bodies, already had a very definite conception of their function, as his name "Leitkörperchen" signifies. That later investigators have invented terms of their own may in some cases have been due to ignorance of Metzner's work, but in others it was due to hesitation to ascribe to the structure in question, without further evidence, the rôle that his term implies. Thus we have beside Leitkörperchen, spindle fibre attachment (many geneticists), Insertionsstelle (Belar and others), attachment body (Schrader), spindle fibre locus (Metz), commissure (Lorbeer), kinetochore (Sharp), and many more.

If my own term, attachment body, merits justification I can only say that I used it because I considered it a descriptive term which does not carry the implication of function.[1] It merely denotes that special region in the chromosome to which the spindle fibre or half-spindle component is connected—as seen in fixed preparations. However, questions of terminology interest me so little that I am quite willing to abandon this nomenclature and accept the term kinetochore, which, even though it ascribes to this body a dynamic rôle of which we have no final proof, is a name that has been used by Sharp (1934) in a book of wide distribution. It thus bids fair to become more generally adopted (as it already has by several other botanists) and therefore to do away with the present unprofitable confusion.

Perhaps my acceptance of Sharp's term is made easier by my belief

[1] Professor C. W. Metz, of the Carnegie Institution, and I have spent a not inconsiderable amount of time in discussing this terminology. His term, *spindle fibre locus*, represents an attempt to preserve a noncommittal attitude concerning the function of the structure involved.

that, even though no conclusive evidence for it is available, the kinetochore is indeed intimately concerned with certain dynamic processes of the chromosome. Such as they are, the foundations of this belief may be stated quite simply. They are that if a kinetochore is visible, it definitely serves as the chromosomal base for the connection between the chromosome and the pole. Further, in following the chromosome through a cell cycle, the kinetochore can be demonstrated cytologically before there is any trace of the half-spindle component (i.e. the connection between chromosome and pole). Therefore even though an important rôle in the mitotic movement of the chromosome be ascribed to the latter, it is not unfair to assume that the kinetochore may be causally involved. As a third support for the belief in a kinetic rôle may be regarded the fact that in the progress from the equator to the pole it is the kinetochore region of the chromosome that leads the way. (But it should not be forgotten that the cases of *Sciara* (Metz, 1933) and *Micromalthus* (Scott, 1936) provide notable exceptions to this rule.) Finally, it seems definitely established in at least some forms that the loss of the kinetochore (be it a natural or experimental effect) results in the omission of the affected chromosome from the division figure and brings about its elimination from the process of orderly mitotic distribution.

In brief, the evidence is not final, but it serves as an adequate basis for a study such as the present paper represents.

Methods

The investigation was made principally on the testes of the amphibian *Amphiuma tridactylum*. The decisive observations were all made on material fixed in Champy's or Benda's fluid, the latter prepared with a very small amount of acetic acid (not exceeding one drop). Other fixations served only a comparative purpose. Heidenhain's hæmatoxylin, strongly extracted, proved the best method of staining for detailed study, but certain of my conclusions, as will appear, involve such mitochondrial stains as those of Benda and Kull. Sections were usually cut as thin as 4 μ, which unfortunately leaves few of the nuclei entire but is advantageous for detailed study. Through Professor E. B. Wilson's kindness I was enabled to study his beautiful slides of the arachnid *Opisthacanthus elatus*, my findings confirming certain of his observations on that animal which pertain to my present work.

First Spermatocyte

In the prophase period, the kinetochore cannot be demonstrated with certainty until the late diplotene and early stages of diakinesis

PLATE I

have been reached. At that time hæmatoxylin preparations, in which the chromosomes have been destained so as to appear an even gray, show the kinetochore as a darker body, not very sharply outlined (Figs. 8, 9, 10). In *Amphiuma*, therefore, the kinetochore at this time shows little or no trace of the achromatic properties that characterize it in many other forms (McClintock, 1933).

In the pachytene and diplotene stages preceding this I have been unable to make a definite identification of the kinetochore, but it is only fair to say that the chromosomes at these phases destain rather unevenly (heterochromatin?) and that it may be a technical difficulty of this sort that is here at fault. Further work involving other techniques is evidently called for.

In the early stage of diakinesis already mentioned, two kinetochores are present in each tetrad. It is to be noted that these are quite independent of each other and that each is associated with a different pair of chromatids (Figs. 8, 9, 10). But though the extreme degree of destaining employed in my technique allows of no detailed analysis of these chromatids, the components of a given pair can be traced in several cases and in none does the line of demarkation between them extend to the kinetochore (Fig. 8). The latter is undivided though it is associated in every instance with two chromatids.

Shortly after this period and at a time when the rapidly condensing, diakinetic tetrads are no longer so closely associated with the nuclear periphery, each of the two kinetochores carried by a tetrad shows a double structure. The two bodies that then characterize each kinetochore have the form of short rods, rather sharply outlined, whose long axis is parallel to the long axis of the chromatids (Figs. 11

PLATE I

Drawings No. 1 to No. 6 made with Zeiss 2 mm. apochromatic objective (n. ap. 1.4), and 10 × ocular. No. 7 to No. 20, inclusive, with 15 × ocular.

1. First spermatocyte metaphase. Not all tetrads in section are shown. Benda fixation and Kull stain. Chromosomes yellow, commissural cup colorless, spindel spherules red, chondriosomes bluish red.

2. Late anaphase of first spermatocyte, with two spindle spherules in each dyad. Benda fixation and hæmatoxylin stain.

3. Interkinesis showing granules. Benda—hæmatoxylin.

4. Early anaphase of second spermatocyte. Separating chromosomes showing that here the kinetochores do not lead the way to the poles. Only one spindle spherule in each daughter chromosome. Benda—hæmatoxylin.

5. Late anaphase of second spermatocyte. Kinetochore now leading poleward movement. Extraneous granules near the poles. Benda—hæmatoxylin.

6. Second spermatocyte division, telophase. Benda—hæmatoxylin.

7. Spermatogonial anaphase in the mouse. Champy fixation, Kull stain. Chromosomes brown, kinetochores and centrioles red, chondriosomes bluish red. Preparation made by A. G. Steinberg.

and 12). Nevertheless, the region of the kinetochore as a whole does not appear to be split, a fact which has some bearing on the analysis of its structure.

With further condensation of the chromatids, these tiny rods seem to shorten until in the fully condensed tetrad they appear as granules or tiny spheres (Figs. 13–15). It is during this process that a light or non-staining region comes to surround them, and I believe that it is this latter area that is the most conspicuous portion of the kinetochore in most other organisms. But whereas it is usually described quite correctly as an achromatic band or commissure that extends transversely through the chromosome, it appears in the *Amphiuma* tetrad more as a notch. Whether this form assumed in *Amphiuma* is in part the result of the bending and torsion of the tetrad I am unable to say, but it seems likely that the explanation is not so simple. Certainly the so-called secondary constrictions, which also appear as transverse achromatic bands, can in the case of *Amphiuma* be traced from an early prophase to final condensation of tetrads, and there is no such alteration in form as that described for the kinetochore (Fig. 15).

Indeed, a very careful examination of well-fixed material indicates that, in the condensed tetrad at least, the form of the achromatic portion of the kinetochore is not so much a notch as a rounded or cup-shaped depression directing its open side toward the pole. The tiny, intensely staining spherules already mentioned adhere to the inner side of this cup, close to its bottom (Figs. 13, 14, 15).

The relation of the kinetochore to the mitotic spindle is quite clearly shown in some of my preparations. White (1935) has recently advanced some evidence convincing him that it is to the commissural or achromatic portion of the kinetochore that the spindle fibre (i.e. the half-spindle component) attaches. But as an examination of my Figure 18 will show, this is not the case in *Amphiuma*. It is from the tiny chromatic spherule that the half-spindle component takes its origin and not from either the commissure or the kinetochore as a whole. It follows therefore that where tetrads are concerned, each half-spindle component has a double source, since two spindle spherules are carried in each commissural cup. That these two, separately arising spindle elements are themselves composed of finer fibrillæ and that they usually join to make a single half-spindle component before reaching the pole is implicit in what I have reported elsewhere (Schrader, 1935). But though the spherules are clearly more directly involved in the association with the spindle, it is equally certain that the commissural cup in which they rest forms an integral part of the

whole apparatus and that it is the commissural cup and the spindle spherules together that constitute the kinetochore.

It is further evident that each of the spindle spherules is associated with a chromatid. The complete tetrad as shown in Figs. 1 and 15 has four spindle spherules, two directed to each pole. Views in which one chromatid is superimposed on the other likewise show superimposition of spindle spherules, as appears from several of my figures. It is only when both chromatids are in face views that the two spindle spherules of a given pair appear as distinct and separate as they actually are.

The further history of the first spermatocyte shows no more than what might be expected from the above. In the actual division, the kinetochore leads the way toward the pole and the chromosome regions farthest removed from the kinetochore are the last to leave the equator. In late anaphases and in the telophase it becomes difficult to make out the detailed structure of the kinetochore, but the two spindle spherules associated with each half of the tetrad continue to stand out sharply until the interkinetic phase is initiated (Figs. 1, 2 and 3).

Interkinesis

The period between the first and second spermatocyte divisions is an aggravatingly difficult one. As the telophase chromosomes become diffuse, there appear many darkly stained granules in the nuclear area (Fig. 3). A little later, when the new nuclear membrane has formed, most of these are peripherally placed. At first I worked on the assumption that these granules are nothing more than spindle spherules which temporarily sever their connection with the chromosomes during this period and become enlarged. But in spite of the wealth of my material, I was unable to make a seriation of stages that might be involved in such a process. The difficulty arises in several ways. Thus certain granules quite extraneous to the chromosomes are present in many telophases of both divisions (Figs. 4 and 5) and these are almost certainly included in the nucleus. Since they vary in number, a mere count is therefore useless. Nor can these last named granules be differentiated from the others on the basis of dimension, for size differences are too small and variable to be of any use. Since my differential stains did not serve to make a distinction either, the hypothesis was temporarily abandoned. It is quite possible that these granules have nothing to do with the kinetochores and that the latter cannot be seen during the interkinesis.

The Second Spermatocyte

The kinetochores of the second division can be definitely recognized shortly before final condensation of the chromosomes. At this time the two chromatids are already quite clearly demarked from each other. Each spindle spherule lies in a light area which in form approximates the typical commissure more closely than does the corresponding region of the first spermatocytes. Side views, however, still show the notch-like configuration (Fig. 20). But although the total number of spindle spherules has not changed between the first spermatocyte telophase and the second spermatocyte metaphase, an essential structural difference is to be observed in the fact that, instead of one kinetochore with two spindle spherules (Fig. 2), we now have two kinetochores each with a single spherule (Fig. 4). In short, the division line between the chromatids now quite definitely extends through the kinetochore. But conditions for detailed study are not as favorable as in the first division and nothing further can be said concerning the detailed structure of the kinetochore at this time.

In the initial chromosome movement toward the pole, the kinetochore does not lead the way as in the first division. Instead, the distal ends of the chromosome move just as rapidly as the kinetochore region, so that it is evident that we are here concerned with what has

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Plate II

All drawings except No. 16 made from preparations fixed with Benda and stained with hæmatoxylin.

8. Tetrad in early diakinesis showing kinetochore as a rather indefinite, dark region.

9 and 10. Slightly later than preceding. Kinetochore becoming more definitely outlined.

11 and 12. Middle phases of diakinesis. Each kinetochore showing two spindle spherules elongated in the long axis of the chromosome. Commissural region beginning to appear as a lighter area.

13. Late diakinesis showing two commissural cups, each with two spindle spherules (one pair superimposed).

14. Metaphase tetrad, one arm cut.

15. Tetrad in metaphase or early anaphase, showing commissural cups, spindle spherules and one secondary constriction.

16. Tetrads in early anaphase, stained with Kull. Kinetochore appearing as red, hemispherical region below a shallow depression in the yellow chromosome. Further differentiation destains commissural part of the kinetochore and leaves spindle spherules as red bodies.

17. Tetrad in early anaphase. Only one kinetochore shown.

18. Dyad in anaphase of first spermatocyte, showing relation of half-spindle component to the two spindle spherules.

19. Polar view of dyad in metaphase of second spermatocyte division.

20. Side view of dyad in second spermatocyte. Each chromatid with one spindle spherule.

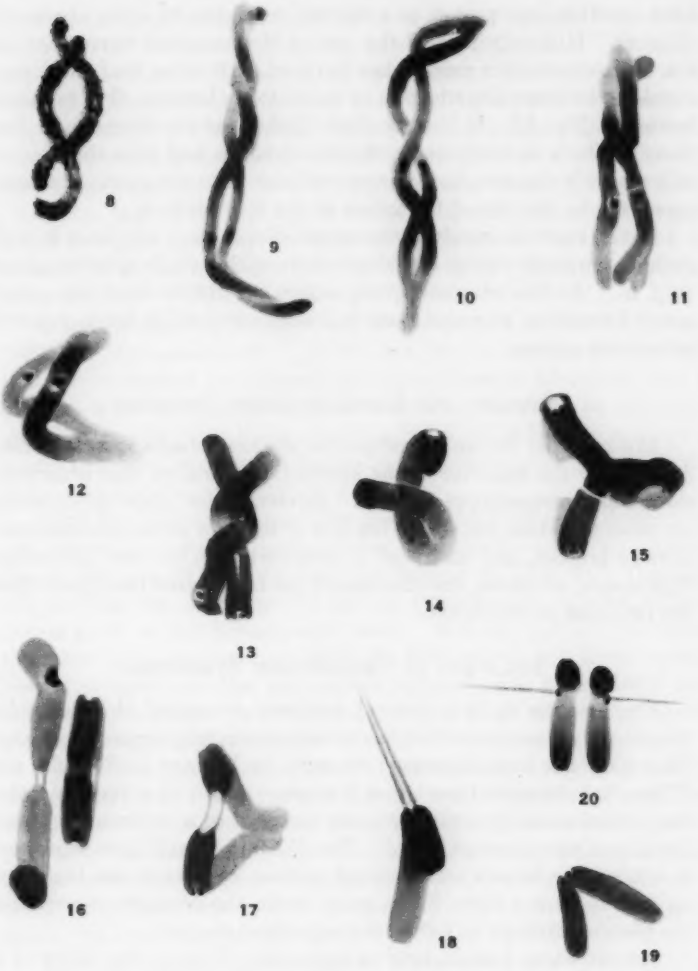

PLATE II

been so often interpreted as a mutual repulsion of sister chromatids (Fig. 4). It is only toward the end of the anaphase movement that the kinetochore once more takes the lead, indicating that the force of repulsion becomes less effective as the distance between the chromatids increases (Fig. 5). It is more than likely that the kinetochore functions similarly in both spermatocyte divisions and that the observed differences in the anaphase movement arise from the special conditions presented by the tetrad formation of the first division.

In any case, the result of the second division is a telophase in which each chromosome carries one kinetochore with a single spindle spherule (Fig. 6). As this telophase group enters the diffuse condition prior to sperm formation, granules, such as are encountered in the interkinesis, once more appear.

Somatic and Spermatogonial Divisions

My material for an investigation of these stages is very limited. Apparently the behavior of the kinetochore parallels that observed in the second spermatocyte division. At metaphase, when the conditions are most favorable for study, the line of division in the chromosome is already present, and each half is provided with its own kinetochore. This means, of course, that division of the latter must take place during the prophase at the latest.

Relation to Chromosome Structure

There seems to be a general tendency to regard the kinetochore simply as a chromomere that has undergone certain temporary changes. That view has been expressed recently by Lorbeer (1934) who says: "There are examples from which it appears clearly that the kinetochore (his commissure) is neither linin nor matrix, but a chromomere exactly like any other chromomere—." The difficulty that I have experienced in tracing the kinetochore through resting and prophases lends some support to such a view, for in many forms the ordinary chromomeres are likewise difficult to follow through these stages.

Nevertheless, I make bold to state that, if this is the origin of the kinetochore, the changes that it has undergone are both extensive and fundamental. Thus the ordinary chromomere has no structure homologous to the commissural cup and the spindle spherule. Again, even hæmatoxylin staining shows that there are considerable differences in the staining reactions, for the spindle spherules remain intensely colored after the rest of the chromosome structure has become decolorized.

But the most striking evidence for a special nature of the kinetochore lies in its response to certain mitochondrial stains. After staining by the Benda method, the chromosomes are brown or amber colored, whereas the kinetochore is violet. After Kull, chromosomes can be brought out as yellow, while the kinetochore region is red or pink. Nor is this red similar to that which characterizes the chondriosomes in the same cell, for the latter have a distinctly more purple cast (Fig. 16). This red region seems to underlie the superficial depression in the chromosome that characterizes the kinetochore and affords additional evidence that the latter is not merely a superficial structure.

A similar staining reaction has been reported already by Metzner (1894) in the salamander. To be sure, by no means all the bodies that he recognized as kinetochores were correctly identified, but his pictures leave little doubt that at least in some cases he was not in error. It is therefore plain that in spite of its ordinarily achromatic properties, the commissural region of the kinetochore will respond to certain special methods of dyeing.

With the Kull stain especially it is possible further to differentiate the kinetochore so as to decolorize the commissural cup entirely, leaving the spindle spherule a dark or intense red (Fig. 1). The result is a striking one, although the optical definition for detailed study is not as good as in a hæmatoxylin stain. But not a doubt can obtain that these staining reactions mark the kinetochore as a highly specialized structure and that the spindle spherule has a decidedly more pronounced reaction to the fuchsin of the Kull stain than has the general commissural substance.

Such staining is encountered not only in *Amphiuma*, but also in the spermatocytes of at least two widely different animals. One of these is the arachnid *Opisthacanthus elatus*, in which Wilson (1931) reported that after the Benda stain the kinetochore (his polar granule) appears a clear violet on the brown background of the chromosome. This is an observation that I can fully confirm and Wilson's drawing (his Fig. 29) shows the conditions perfectly.

The other animal is the common mouse (*Mus musculus*), in which the Kull stain brings out the kinetochore as a red body while the chromosome is yellow (Fig. 7). The staining reaction in this case is not a difficult one, and several of my students have been successful in obtaining it (A. Greenberg, M. H. Slud, A. G. Steinberg, among others).

It is, however, to be admitted that the size of the chromosome as well as the kinetochore in both *Opisthacanthus* and *Mus* are such that a

detailed study comparable to that made in *Amphiuma* becomes difficult or even impossible. Nevertheless, they serve to show that my conclusions concerning the nature of the kinetochore in *Amphiuma* probably have a broader application. Whether it is always of the form that I have described is another matter. Thus it may be pointed out that Sharp (1929), Nebel (1935), and White (1935), among others, picture the spindle spherule as terminating a fine thread. Nebel states, however, that such a form is probably the result of acid fixation and that as the acid in the fixing fluid is decreased, the thread becomes shorter and finally disappears. I attempted to test this conclusion, but though in a sense my own findings support Nebel (since in them the absence of a thread is correlated with little or no acid in the fixing fluid), I was unable to obtain preparations in which the spindle spherule shows any thread. Indeed, after strongly acid fluids my material did not show the kinetochore at all.

The Kinetochore in the Mitotic Cycle

It will be noticed in my drawings of *Amphiuma* that after extreme differentiation the centriole is so small as to be difficult to observe. I have found this to be the case even after the Benda stain, which in my experience brings out the centriole better than any other. In *Opisthacanthus*, on the other hand, the centriole is quite conspicuous after this stain, taking on a violet color that is much closer to that of the kinetochore than of the chondriosomes. Even without the rest of my evidence, these reactions would prevent me from proposing a chondriosomal nature for the kinetochore.

On the other hand, a relationship between the centriole and the kinetochore might well be considered. To be sure, the staining reaction just referred to offers but a fragile basis, but some additional slight evidence may be derived from certain observations of Belar (1929). He found that in the live chromosomes of *Chorthippus* the attachment region (the kinetochore) of the chromosome appears as a clear spot, and I can affirm this for another species of grasshopper. Unfortunately opportunity was lacking to study the living cells of *Amphiuma* with this point in mind. Now, a clear or glassy character has also been reported for the living centrioles in at least two forms. These are *Œcanthus* (Johnson, 1931) and *Drosophila melanogaster* (Huettner and Rabinowitz, 1933), and again I can confirm the observation, this time on material prepared by these workers themselves. However weak, the evidence is therefore not against the hypothesis of some sort of a relationship between centriole and kinetochore.

If there is such a relationship it may well be that in it lies the key to the puzzle of mitotic movement. But with so little evidence I have no intention of entering this old battlefield of hypotheses. I mention it chiefly in order to state that the idea of such a relation underlies the conclusions of some of my fellow workers, whether or not they are conscious of it. I am referring especially to certain convictions concerning the bouquet stage in the meiotic prophase, where, it will be remembered, the leptotene threads show a definite orientation toward a point on the nuclear periphery usually close to the centriole. It is very natural to conclude that this movement must be attributable to some type of rapport between centriole and kinetochores, and such an assumption is implicit in the writings of several investigators, notably Janssens (1924).

Confessing to a certain amount of sympathy with the view, I am nevertheless constrained to point out that there is some weighty evidence to the contrary. This derives from the very careful work of such investigators as Gelei (1921) and Mohr (1916). Mohr, for instance, has shown conclusively that in the leptotene polarization of the long chromosomes of *Locusta viridissima*, where the kinetochore must be close to median in position, it is the ends that are drawn toward the centriole. In fact the kinetochore region is in this phase as far as possible removed from the point of centralization.

By this same token it becomes questionable whether the polar granule of Pinney (1908) and Wenrich (1916) is to be regarded as identical with the kinetochore. Wenrich himself states (p. 64) that the polar granule appears at the same end of each chromosome to which the spindle fibre attaches, but he by no means commits himself to the view that it is the polar granule that is instrumental in this process of attachment. Indeed, he could hardly do so when it is considered that within a few sentences of the above he notes his agreement with the findings of Miss Pinney (1908) that in certain chromosomes similar granules also occur at the end of the chromosome opposite that to which the spindle fibre attaches. On page 69 he declares that the chromosome pair B "is characterized by the presence of large and well-marked polar granules at both ends." Similarly, although the "granules proximaux" that Janssens (1924) describes in the metaphase chromosomes are certainly kinetochores, it is by no means certain that these are identical with the granules to which he gives the same name in the bouquet stage. In view of the evident importance of these bodies a cautious attitude seems warranted.

Cytogenetic Aspects

That the kinetochore plays an important rôle in the cell cycle is sufficiently clear from its effects on crossing-over and chiasma formation. In Darlington's hypothesis (1932) concerning the meiotic mechanism it is the kinetochore that is accountable for the fact that division of the tetrads always occurs into pairs of chromatids. Such effective singleness of the kinetochore also would support Darlington's chiasma type and terminalization hypotheses "which demand that the identical chromatids should remain together at the spindle attachment, although nowhere else."

Nebel has recently (1935*a*) questioned this rôle that Darlington ascribes to the kinetochore. His doubts stem from his discovery that in the first meiotic anaphase of *Tradescantia reflexa* each pair of chromatids (in the sense of Darlington) is provided with four Zugfasern where the presence of even two would provide a definite objection to Darlington's hypothesis.

Now, "Zugfasern" (Belar's traction fibres) are regarded by Nebel as an attenuated form of the kinetochore, which is assumed during meta- and anaphase. Hence the presence of two or more Zugfasern indicates that the kinetochore has divided and therefore Darlington's singleness of that body does not hold.

In this connection I should like to point out again that the evidence for *Amphiuma* strongly indicates that the kinetochore is a compound body. In *Amphiuma* also, the first anaphase shows two spindle spherules for every pair of chromatids, but it is clear that these are not free to move independently of each other. They are held together by the undivided commissural cup. Since I regard Nebel's Zugfasern as equivalent to my spindle spherules, I do not consider that his demonstration of their division and subdivision (which I do not question for one moment) carries with it the proof that the kinetochore as a whole has also divided. In short, Darlington's thesis does not necessarily collapse in the face of Nebel's fine evidence.

On the other hand, Nebel's implication that at metaphase each chromatid (or chromonema) is provided with a spindle spherule is well substantiated by my findings. And further, the presence of four spindle spherules for each *Amphiuma* tetrad, whereas eight are to be encountered in the tetrad of *Tradescantia*, can therefore only support Nebel's conclusion (1935*b*) that there is a definite difference between animals and plants with respect to the number of chromonemata involved during meiosis (Nebel reports four for the orthopteran *Dissosteira* and eight for *Tradescantia*).

SUMMARY

1. The kinetochore is a compound body composed of a commissural region and a spindle spherule, so far as univalent chromosomes are concerned.

2. The half spindle component connects with the chromosome through the spindle spherule.

3. Already during the diakinetic phases of meiosis, each tetrad has two kinetochores which are distinct and separate from each other. Each of these is provided with two spindle spherules, so that each tetrad carries a total of four spindle spherules.

4. In the first spermatocyte division, the kinetochores lead the way to the poles. The dyads resulting from this division each show two spindle spherules until the resting stage is reached.

5. At metaphase of the second spermatocyte, each dyad is already sharply differentiated into two chromatids or univalent chromosomes. Each of these carries a kinetochore provided with a spindle spherule.

6. Aside from its structure, various staining reactions mark the kinetochore as an organ quite distinct from the ordinary chromomere.

7. The possible relation of the kinetochore to cell mechanics is discussed, and certain cytogenetic aspects are considered.

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